Human dendritic cell development from induced-pluripotent stem cells

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Abstract

Dendritic cells (DCs) are a myeloid cell type specialised in processing and presenting antigens to T cells and they represent essential mediators of innate and adaptive immunity. In humans, DCs represent a very rare population of cells (0.02-0.8 % of leukocytes in the blood) and this aspect limits the ability to study human DCs subsets and their potential application in cell therapy-based translational studies. In order to overcome these limitations and provide a reliable system to differentiate human DCs from an inexhaustible source of cells, this work aims to develop a new experimental system to generate DCs from induced pluripotent stem cells (iPSCs), both in *in vitro* and *in vivo* settings. In this regard, a culture system based on engineered mouse stromal cells was established to support human DC development through the induction of a definitive hematopoietic program in iPSC-derived hematopoietic progenitors.

The differentiation of human DCs from cord blood-derived hematopoietic progenitors was achieved and the heterogeneity of *in vitro* generated CD1c+ cells was investigated. Moreover, the ability of Flt3L to influence the early stages of hemato-endothelial specification was unravelled.

Ultimately, this approach will be applied to genetically modified iPSC using CRISPR/Cas9 system. Candidate genes were identified and targeted in iPSCs in order to assess their influence on i) manipulate subsets commitment through transcriptional networks perturbation (i.e. IRF8, IRF4, ID2) and ii) manipulate DCs immunogenicity by targeting immune checkpoints (i.e. PD-L1).

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Declaration

I declare that I have personally prepared this thesis, and that the work included is my own unless otherwise stated. All information sources included in this thesis are referenced accordingly.

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Abbreviations

DC: dendritic cell

CD: cluster of differentiation

APC: antigen-presenting cell

Ag: antigen

CDP: common dendritic cell progenitor

TLR: Toll-like receptor

MHC: major histocompatibility complex

Flt3L: Fms-related tyrosine kinase 3 ligand

CCR: C-C chemokine receptor

BDCA: blood dendritic cell antigen

MPS: mononuclear phagocyte system

GM-CSF: granulocyte macrophage colony stimulating factor

TNF: tumor necrosis factor

M-CSF: macrophage colony stimulating factor

HSC: hematopoietic stem cell

cMoP: common monocyte progenitor

LC: Langerhans cell

PBMC: peripheral blood mononuclear cell

IFN: interferon

IL: Interleukin

Batf3: basic leucine zipper transcriptional factor ATF-like 3

IRF: interferon regulatory factor

PolyI:C: polyinosinic-polycytidylic acid

HCV: hepatitis C virus

CXCL: C-X-C motif chemokine ligand

CTL: cytotoxic T lymphocyte

PD-L1: programmed death-ligand 1

SLAN: 6-sulfo LacNAc

CLP: common lymphoid progenitor

CMP: common myeloid progenitor

MDP: monocyte/dendritic cell progenitor

GMP: granulocyte-macrophage progenitor

RTK: receptor tyrosine kinases

ID: inhibitor of DNA-binding

DCML: dendritic cell, monocyte, B and NK lymphoid

NK: natural killer

CyTOF: time of flight mass cytometry

MSPC: mesenchymal stem and progenitor cell

SCF: stem cell factor

TPO: thrombopoietin

EC: endothelial cell

CXCR: C-X-C chemokine receptor

BM: bone marrow

RANK: receptor activator of nuclear factor NF-kB

DKK: dikkopf homolog 1

SFRP1: secreted frizzled-related protein 1

WIF: Wnt inhibitor factor 1

TAA: tumor-associated antigen

moDC: monocyte-derived dendritic cell

moMacs: monocyte-derived macrophage

NSCLC: non-small cell lung cancer

HNSCC: head and neck squamous carcinoma

FDA: food and drug administration

HSPC: hematopoietic stem and progenitor cell

XCR: X-C motif chemokine receptor

SCID: severed combined immunodeficiency

BAC: bacteria artificial chromosome

AGM: aorta-gonad-mesonephros

CS: Carnegie stages

EMP: erythro-myeloid progenitor

LMPP: lymphoid-primed multipotent progenitor

P-Sp; para-aorta splanchnopleura

BL-CFC: blast colony-forming cell

EHT: endothelial-to-hematopoietic transition

ESC: embryonic stem cell

PSC: pluripotent stem cell

iPSC: induced-pluripotent stem cell

HE: hemogenic endothelium

IAHC: intra-aortic hematopoietic cluster

CFU-S: colony-forming unit spleen

LTR: long-term reconstitution

NSG: NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ

YS: yolk sac

DLP: dorsolateral plate

PLM: posterior lateral mesoderm

BMP: bone morphogenetic protein

Shh: Sonic-hedgehog

CFC-C: colony-forming unit culture

NO: nitric oxide

VEGF: vascular endothelial growth factor

ACE: angiotensin converting enzyme

DLL: delta-like

KDR: kinase insert domain receptor

TGF: transforming growth factor

FGF: fibroblast growth factor

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells

HOX: homeobox

SOX: SRY-related HMG-box

PDFGR: platelet-derived growth factor receptor

EB: embryoid bodies

HUVEC: human umbilical vein endothelial cells

LPS: lipopolysaccharide

CRISPR: clustered regularly interspaced short palindromic repeats

Cas9: CRISPR associated protein 9

IRES: internal ribosome entry site

GSI: gamma-secretase inhibitors

ELISA: enzyme-linked immunosorbent assay

sgRNA: single guide RNA

INDEL: insertion deletion

TIDE: tracking of indels by decomposition

Chapter 1 Introduction

1 Human dendritic cell development and *in vitro* modelling

1.1 Mouse dendritic cells: brief history and subset characterisation

Dendritic cells (DCs) were first described by Ralph Steinman and Zanvil Cohn in the early 1970s by observing and describing the heterogeneity of adherent cells isolated from mouse spleen[1,2]. In the seminal paper introducing the concept of dendritic cells for the first time, Steinman and Cohn observed *"in addition to mononuclear phagocytes, granulocytes, and lymphocytes, [we noticed] a large stellate cell with distinct properties from the former cell types"* and they first named these cells dendritic cells, based on their specific morphology[1]. A few years later, the idea that dendritic cells represent an independent hematopoietic lineage was further supported by demonstrating that DCs have the unique ability to initiate a strong and specific T cell response in both syngeneic and allogeneic mixed leukocyte reactions[3–5]. This contributed to the identification of DCs as the most potent antigen presenting cells (APCs) of the immune system, and firmly established the role of DCs as a bridge between innate and adaptive immunity. Indeed, DCs can sense peripheral tissues, capture environmental and cell-associated antigens (Ag) and migrate to lymph nodes, where they process and present phagocytosed antigens to T cells, establishing an Ag-specific immune response. Moreover, the identification of a clonogenic DC progenitor, the common dendritic cells progenitor (CDP), in the mouse bone marrow, which has the potential to differentiate exclusively to dendritic cells, further consolidated the notion that DCs represent a unique cell type distinct from all the other leukocytes[6–8]. Finally, fate mapping experiments further confirmed the existence of DCs as a specific lineage[9,10].

Two main subsets of dendritic cells have been described in mouse lymphoid organs: plasmacytoid DCs (pDCs) and conventional DCs (cDCs).

pDCs represent a small subset of DCs present in blood and lymphoid organs. pDCs morphologically resemble plasma cells and they are characterized by their ability to produce massive amounts of type I interferon upon recognition of foreign nucleic acids by the Toll-like receptors 7 (TLR7) and 9 (TLR9), suggesting an important role for pDC during viral infection[11]. Like cDCs, pDCs arise from a common dendritic cells progenitor (CDP), and they can prime T cells against viral antigens upon stimulation[11]. As compared to conventional DCs, steady state pDCs express lower levels of major histocompatibility complex II (MHCII) and CD11c, and they can be identified by the co-expression of several surface markers, such as CD45RA, B220 and PDCA1.

cDCs represent the subset originally described by Steinman and Cohn, characterized by the enhanced ability to sense peripheral tissues and trigger a specific T cell response. This unique ability is the result of several specific features of cDC, consisting of i) their critical localization in non-lymphoid tissues and spleen marginal zone ii) their ability to process and present antigens via MHCI and MHCII[12–14] iii) their superior ability to migrate to the lymph nodes and prime T cell responses[15,16]. cDC can be identified in blood, lymphoid and non-lymphoid organs, and they are phenotypically defined by the expression of CD45, MHCII and CD11c and the lack of markers specific for T cells, B cells, NK cells, granulocytes and erythrocytes. Moreover, even if they share a consistent number of markers with macrophages, cDCs can be successfully distinguished by the expression of the tyrosine kinase receptor fms-like tyrosine kinase 3 (Flt3/CD135/Flk2), their reliance on Flt3L for development[17] and their independence from CCR2 which distinguish them from monocyte-derived cells[18].

Conventional DCs can be further subdivided in two subsets, based on the expression of specific surface markers, which can vary depending on their lymphoid and non-lymphoid tissue localization. Lymphoid tissue-resident cDCs consist of two subsets, CD8α+ cDCs and CD11b+ cDCs, which correspond to CD103+ cDCs and CD11b+ cDC in non-lymphoid organs.

Mouse lymphoid CD8α+ and non-lymphoid CD103+ cDC subsets share their transcriptome profile, development requirements and the expression of subset-specific markers such as XCR1, Clec9A (DNGR1), CADM1 (Necl2) and higher expression of CD24, as compared to CD11b DCs. Mouse CD8α+/CD103+ DCs are characterized by their superior ability to cross-present exogenous antigens to CD8+ T cells via MHC class I molecules[12,19,20], suggesting functional specialization of the two cDC subsets.

Mouse CD11b+ DCs are also present in both non-lymphoid and lymphoid organs, where they additionally express CD4, along with other surface markers specific for this subset, such as CD172a (SIRPα). Differential expression of endothelial cell-specific adhesion molecule (ESAM) suggests heterogeneity of these cells, which can be further separated in two subpopulations of $ESAM^{hi}$ CD11b+ and $ESAM^{lo}$ CD11b+ cells[21,22]. Mouse CD11b+ DCs are generally considered poor cross-presenters *in vivo* when compared to CD8α+/CD103+ DCs, but they are characterized by a superior ability to prime CD4+ T cells via MHC class II presentation[23,24].

1.2 Human dendritic cells and the mononuclear phagocyte system

Human dendritic cells are defined as antigen-presenting leukocytes with allo-stimulatory capacity, characterised by the lack of lineage-specific markers (CD3, CD14, CD15, CD16, CD19, CD20, CD56) and the expression of MHC class II.

Counterparts of mouse DC subsets have been described in human based on the comparative analysis of their transcriptome along with the identification and assessment of subset-specific functions. Like in mouse, human DCs in blood and lymphoid organs can be divided in two main subsets: CD11c- plasmacytoid (pDC) and CD11c+ conventional DCs (cDC). Plasmacytoid DCs can be further defined as CD33- CD123+ CD45RA+ BDCA2/CD303+ BDCA4/CD304+ cells, whereas conventional DCs consist of at least two subsets: BDCA3/CD141+ DCs and BDCA1/CD1c+ DCs, corresponding to the mouse CD8α+ and CD11b+ cells, respectively[25–28]. Moreover, several markers are conserved between human DCs and their mouse counterparts, such as XCR1, CADM1 (Necl2), Clec9a (DNGR1) and Flt3 in BDCA3/CD141+ DCs, CX3CR1, Flt3 and CD172a (SIRPα) in BDCA1/CD1c+ DCs and CD123 and CD45RA in pDC.

Dendritic cells, both in human and mouse, are one of the lineages composing the mononuclear phagocyte system (MPS). The mononuclear phagocyte system was first proposed by van Furth in the late 1960s, to define a group of leukocytes with specific abilities to process and present antigens[29]. The MPS includes monocytes, macrophages and dendritic cells; closely related cell types whose existence as distinct lineages has represented a source of controversy for many years[30,31]. Indeed, human monocytes can be induced to differentiate into dendritic-like cells (moDCs) and macrophage-like cells (moMacs) upon exposure to cytokines such as GM-CSF, IL4 and TNFα or M-CSF, respectively[32–35]. However, it is now clear that this *in vitro* approach, albeit having provided an invaluable tool to study rare and not easily accessible human cells, does not faithfully recapitulate the developmental processes occurring *in vivo*. The hematopoietic stem cell (HSC)-independent embryonic origin of tissue-resident macrophages has been formally proven in the mouse model[36–41], and human tissue-resident macrophages share the same properties. In this regard, during HSC transplantation dermal macrophages appeared to be of recipient origin for a prolonged amount of time when compared with

dermal DCs[42], and Langerhans cells have been reported to be of donor origin up to ten years after limb transplantation[43], suggesting that human macrophages are capable of self-maintaining or are long lived and are independent from circulating HSC-derived monocytes. Furthermore, circulating monocytes and all cDC subsets were absent in patents with a mutation in the transcription factor GATA2, whereas Langerhans cells and macrophages were present at normal numbers in the skin and lungs. These observations supported the independent development of Langerhans cells and tissue-resident macrophages from monocytes and DCs[44]. Unlike tissue-resident macrophages, human monocytes and DCs both originate from HSCs and they are constantly replenished by specific progenitors residing in the bone marrow [45]. Independent progenitors with restricted potential for either DCs (CDP)[45] or monocytes (cMoP)[46] have been identified in human bone marrow, underlining the distinct origin of these cells. Moreover, transcriptional analysis further confirmed the separation of DCs from monocytes and macrophages both in humans and mice[26,47–49], in a more unbiased fashion not based only on the expression of partially shared, and therefore potentially misleading, surface markers. Moreover, further evidence has been provided in the mouse model, where a precursor for moDCs has been identified within the Ly6C+ monocytes compartment, characterised by the ability to give rise to CCR2-dependent, Flt3L-independent moDC, not labelled in Zbtb46-cre fate mapping experiments and strongly dependent on high levels of PU.1[50].

A further source of complexity, which characterised the MP system for the past decades, was the absence of a unified and consistent nomenclature to describe and define the different members of the system. For instance, the partial overlap of the surface markers used to identify different cell types as well as their shared functions in specific contexts, can greatly affect the ability to unequivocally identify the lineages involved in a certain biological process. Likewise, the phenotypic differences observed

in each cell type within different tissues in the same organism as well as among different species can easily represent a source of further confusion. Recently, a novel approach has been proposed to define a unified nomenclature for both human and mouse MPS, based on the ontogeny of these cells as opposed to their function and/or phenotype[51] (Figure 1-1). According to this new system, MPS cells have been first divided in three main groups based on their ontogeny: macrophages when embryonically derived, monocyte-derived cells when cMoP-dependent, and dendritic cells when CDP-derived. Furthermore, dendritic cells have been classified in three main subsets: cDC1, cDC2 and pDC. Conventional DC1 subset includes the previously described human BDCA3/CD141+ cells from both lymphoid and non-lymphoid tissues, as well as mouse CD8α+ lymphoid and CD103+ CD11b- non-lymphoid dendritic cells. Likewise, cDC2 subset can be used to define human BDCA1/CD1c+ cells in the blood and lymphoid organs, the BDCA1/CD1c+ CD1a+ cells present in non-lymphoid organs and the mouse lymphoid and non-lymphoid CD11b+ cDCs. Based on this nomenclature, all the cells derived from monocytes, even if they partially share their phenotype or function with DCs (e.g. CD14+ DCs, inflammatory DCs) and/or macrophages, should be considered a distinct lineage and referred to as monocyte-derived cells (blue panel in Figure 1-1). This system will be adopted in this manuscript.

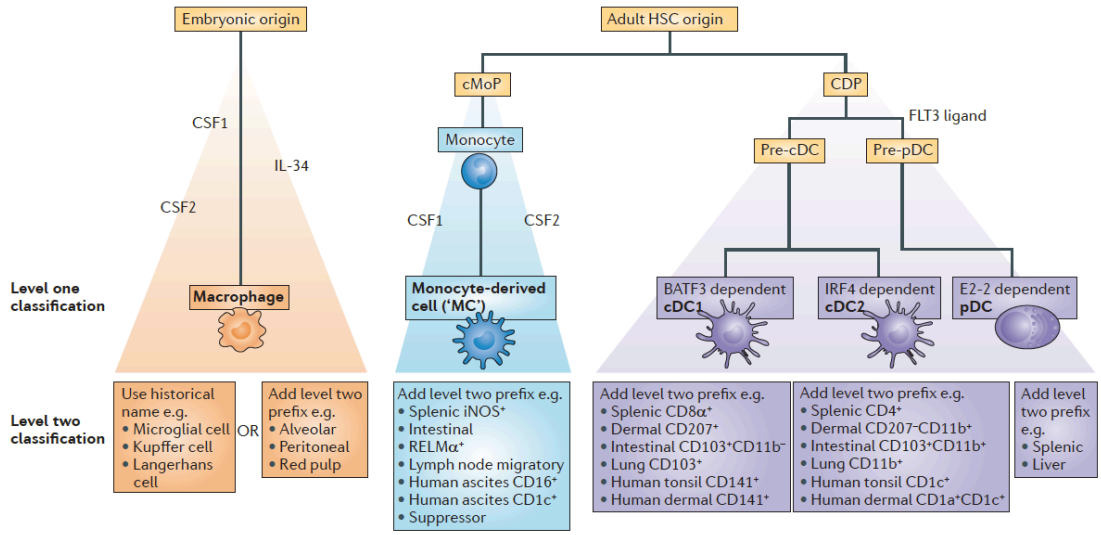


Figure 1-1. A unified nomenclature for MPS classification (from Guilliams et al., 2014[51]). Three main groups of cells can be identified based on their ontogeny: embryonic-derived macrophages (orange), monocyte-derived cells (blue) and CDP-derived cells (purple). CDP-derived cells were further divided in three subsets (cDC1, cDC2 and pDC, i.e.) based on their subset-specific transcription factor dependency.

1.3 Anatomical classification of human dendritic cells

Human dendritic cells can be classified based on their anatomical localization in lymphoid organs- "resident" cDCs, and non-lymphoid tissues- "migratory" cDCs.

1.3.1 Non-lymphoid tissue cDC1 and cDC2s

Several DC subsets can be identified in non-lymphoid tissues. These cells are defined as "migratory" cDCs for their ability to acquire antigens, mature and migrate to the lymph nodes via afferent lymphatics in a CCR7-dependent fashion. In the lymph nodes, "migratory" cDCs can present their antigen to T cells to initiate an antigen-specific adaptive response. Two main subsets of cDCs, BDCA3/CD141+ cDC1 and BDCA1/CD1c+ CD1a+ cDC2 can be detected in human skin, liver, lung and intestine, co-existing with other macrophages and monocyte-derived cells. Additional subsets have also been described in mucosal tissues. In the skin, Langerhans cells (LC) and CD14+ DCs can be identified in the epidermis and dermis, respectively. These cells have been initially included in the dendritic cell lineage based on their ability to spontaneously migrate *ex vivo* from tissue explants[52–55] and to prime Th1, Th2 and Th17

responses *in vitro*[56,57]. However, the presence of LC in patients harbouring a GATA2 mutation preventing cDC and monocyte development[44], as well as the detection of donor LC after ten years in transplanted limbs[43] defined LC as a distinct lineage from cDCs, a concept further supported by mouse evidences describing LC ability to renew in the skin[58] and their potential embryonic origin[37]. Likewise, transcriptome analysis of CD14+ DCs strongly suggests a monocytic origin for these cells, therefore excluding them from the cDC lineage[59].

Human small intestine cDCs differ from other non-lymphoid tissue cDCs, in that the integrin CD103 is expressed in both BDCA3/CD141+ cDC1 and BDCA1/CD1c+ cDC2, which also express SIRPα/CD172a. Moreover, a third subset can be identified as CD103- SIRPα/CD172a+ cells, which was found to cluster with monocytes in transcriptional profiling analysis, suggesting their monocytic origin[28]. Finally, an additional subset termed as "inflammatory" DC (InfDC) has been described in human inflamed tissues, which has been characterised as a monocyte-derived population[60,61].

1.3.2 Blood and lymphoid organ DCs

In the blood, human dendritic cells represent approximately 1% of the circulating peripheral blood mononuclear cells (PBMCs), and they are classified in three subsets: BDCA3/CD141+ cDC1, BDCA1/CD1c+ cDC2 and BDCA2/CD303+ CD123+ pDCs. More recently, a new classification has been proposed based on single-cell RNA-seq experiments[62], which will be described in detail in a dedicated section of this thesis. Through the circulation, human DC can seed all lymphoid organs, such as spleen, lymph nodes and tonsils, where they reside for their entire life playing a role in tissue homeostasis and inflammation. These cells are defined as "resident" DCs, as opposed to the "migratory" DCs found in non-lymphoid tissues. Resident DCs represent the only subsets present in lymphoid organs lacking afferent lymphatics[26,63,64], whereas they can

be distinguished by their lower expression of MHCII and higher expression of CD11c from their "migratory" counterparts also present in the lymph nodes.

1.4 Human DC subsets in blood and lymphoid organs

Human blood and lymphoid-tissue resident cDCs represent the most relevant compartment for the aims of this thesis, and therefore they will be extensively discussed in the next sections of this chapter, focusing on their phenotype, function, transcription factor dependency and growth factor requirements (Table 1-1). Moreover, two further sections will be dedicated to the description of additional DC subsets recently identified in human blood[62] and to the characterisation of human inflammatory DCs.

Table 1-1. Human dendritic cell subsets in blood and lymphoid organs

DC subset	Phenotype	TLR response	Cytokines secretion	Frequency in PBMCs
cDC1	BDCA3/CD141$^+$ DNGR1/Clec9A$^+$ XCR1$^+$ Flt3/CD135$^+$ CD11c$^+$	TLR3	IFN-λ	0.02-0.06%
cDC2	BDCA1/CD1c$^+$ SIRPα/CD172a$^+$ Flt3/CD135$^+$ CD11c$^+$ CD14$^-$ CD16$^-$ CD206$^-$	TLR4 TLR8	IL-12p70 TNFα IL-6 IL-1β IL-23 (skin)	0.3-0.8%
pDC	BDCA2/CD303$^+$ CD123$^+$ CD45RA$^+$ CD11c$^-$ Flt3/CD135$^+$ CD33$^-$	TLR7 TLR9	IFN-α/β	0.3-0.8%
moDC (inflammation)	BDCA1/CD1c$^+$ SIRPα/CD172a$^+$ Flt3/CD135$^-$ CD11c$^+$ CD14$^+$ CD16$^-$ CD206$^+$	TLR4 TLR8	TNFα TGFβ IL-1β IL-6 IL-23 IL-10	-

1.4.1 Human DC1 subset

Human DC1s represent the rarest subset of DCs both in human and mouse, and they account for 0.03% of total PBMCs in blood. cDC1s were originally named as BDCA3/CD141+ DC, based on their expression of thrombomodulin (CD141). However, CD141 does not represent a specific marker for cDC1s and other members of the MPS such as monocytes and *in vitro* cultured cDC2 have been reported to up-regulate CD141 under certain conditions. Therefore, additional membrane proteins have been identified as more reliable markers to identify human and mouse cDC1s, including the C-type lectin-like receptor Clec9A[65,66] and the chemokine receptor XCR1[67]. Consistent with the mouse system, human cDC1 also express fms-like tyrosine kinase 3 (Flt3/CD135) and strongly depend on Flt3L for their development both *in vitro*[68,69] and *in vi*vo[70]. The transcriptional regulation of human cDC1 is less clear compared to their mouse counterparts. Like in mouse, cDC1s express both interferon regulatory factor 8 (IRF8) and the transcription factor Batf3. Moreover, patients harbouring IRF8 mutations showed a dramatic reduction in all DC subsets including cDC1, suggesting a key role of IRF8 in DC development[71]. Conversely, conflicting results were reported for Batf3, which plays a critical role in mouse cDC1 development and appeared to be required for human cDC1 *in vitro*, but not *in vivo* in humanised mice[66].

Expression of TLR1, TLR3, TLR6, TLR8 and TLR10 has been reported in human DC1 either at mRNA or protein level[25,63,72–74]. TLR3 activation by PolyI:C (dsRNA) or HCV infection induces type III interferon (IFN-λ) production by cDC1[75–77], a feature conserved in the mouse system, whereas no cytokine production has been reported in response to TLR8 activation in human cDC1[73,78]. Moreover, human cDC1 can efficiently secrete TNFα and CXCL10, but very low IL12, a major difference with their mouse counterparts, which are known to be the most effective producers of IL12[26,68,72,73].

Like in mouse, human cDC1 are capable of inducing CD8+ CTL response against tumors and viral infections[67,68,72,79].

1.4.1.1 Human DC1 in T cell responses

The most peculiar function of mouse cDC1 is their superior ability to cross-present various forms of antigens to CD8 T cells via MHCI[80]. This aspect is not fully conserved in humans, where all blood and lymphoid organs DC are capable of cross-presenting antigens to CD8 T cells[81], and have comparable efficiency in soluble antigen cross-presentation regardless of their activation state[60,63,64]. However, human cDC1 appeared to be more efficient than other subsets in cross-presenting dead cell-derived antigens upon TLR3 stimulation and antigens delivered via CD205 targeting[26,60,67,72,79,82,83]. Several features of human cDC1 are responsible for their efficient cross-presentation to CD8+ T cells. TLR3 and Clec9A, are known to be important in cross-priming[82,84–87]. Moreover, cDC1s express the chemokine receptor XCR1[67,79], whose ligand XCL1 is produced by activated CD8+ T cells, inducing the recruitment of DC1s and supporting the establishment of a strong adaptive response[88]. Finally, mouse cDC1s have been shown to play a crucial role in anti-tumor immunity and they accumulate in regressing tumors[89,90]. Supporting a similar role for cDC1s in human, a positive correlation of the expression of transcript associated to cDC1 and patient outcome has been reported[89].

In humans, both cDC1s and cDC2s can induce allogeneic CD4+ T cells proliferation *in vitro*[28,63,72,73,91]. In contrast with mouse data, human cDC1s do not show a subset-specific ability to induce Th1 polarization of CD4 T cells, and both cDC1s and cDC2s can efficiently induce a Th1 response when activated by a variety of stimuli[64,72,73,92]. Moreover, human cDC1s appeared to be superior to cDC2s in inducing Th2 responses[91], in contrast with the mouse system, where CD11b+ cDC2s are known to be the most efficient subset promoting Th2 polarisation. However, these observations are based on *in vitro* assays using human allogeneic CD4 T cells, and different results have

been reported when an Ag-specific autologous response was measured, suggesting that cDC1s and cDC2s are equally efficient in processing and presenting antigens to CD4 T cells[72,83]. Even if a role in tolerance induction has been proposed for mouse cDC1s[93], a similar function for human CD141+ cDC1 has not yet been reported.

1.4.2 Human cDC2 subset

Human cDC2s can be identified in lymphoid and non—lymphoid tissues as well as in the blood, where they represent approximately 0.3% of total PBMCs. The phenotype of human cDC2s in blood and lymphoid tissue can be defined as CD11c+ HLA-DR+ CD14- CD16- CD2+ CD1c+ CD45RO+ SIRPα/CD172a+ cells[60,63,94,95]. In addition, other markers have reported to be expressed in these cells, such as CD1a in the skin and CD103 in the intestine, highlighting tissue-specific discrepancies in the cDC2 phenotype. Human cDC2 phenotype closely resembles the phenotype described for inflammatory monocyte-derived DCs and therefore a re-evaluation of their function, especially during inflammation, may be needed[60]. Moreover, heterogeneity of human cDC2s has been recently described based on their phenotype and transcriptomic analysis, suggesting the existence of two independent subsets within the cDC2 population[62,96], as previously described in mouse[21,22]. Human cDC2s also express Flt3 and accordingly, Flt3L expands this subset *in vitro* and *in vivo*[34,70,97–99].

The transcriptional regulation of human cDC2s is not fully elucidated, but the specific expression of the interferon regulatory factor 4 (IRF4) may suggest its potential involvement in controlling cDC2s development, consistent with the mouse system[100]. However, it has been shown that autosomal dominant mutation of IRF8 in patients induces an abnormal phenotype in cDC2s, without affecting the cDC1s, suggesting a different transcriptional regulation of DC development between human and mouse[71]. Furthermore, cDC2-deficient patients appeared to be more susceptible to mycobacteria

infections[71], which is consistent with the specific ability of cDC2s to present glycolipid antigens and trigger responses to mycobacteria by virtue of CD1c expression[101].

The expression of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR8 has been reported in human cDC2 either at mRNA or protein level[25,63,72–74]. Activation of TLR2 and TLR8 can trigger IL12-p70 secretion in blood and lymphoid cDC2s, making them the best IL12 producers among all DC subsets[60,78,102]. Moreover, blood and tissue cDC2s can also secrete IL1β, TNFα, IL8 and IL10 in response to specific stimulation[56,73], and IL23 production in skin, lung and intestine by cDC2s has been reported in response to *Aspergillus fumigatus* exposure or TLR8 stimulation[92,103].

1.4.2.1 Human cDC2s in T cells responses

Human cDC2s from spleen, lymph node, tonsil, skin and lung of humanized mice are capable of cross-presenting antigens to CD8+ T cells, in some cases with the same efficiency of cDC1s, depending on the source of antigen and the kind of stimulation[60,63,64,104]. Moreover, blood activated cDC2s can induce cytotoxic T lymphocytes (CTL) differentiation and trigger higher levels of granzymes expression as compare to human cDC1s, possibly due to their ability to secrete IL12-p70[78]. Furthermore, lung cDC2s have been associated with the induction of CD103 acquisition by CD8+ T cells, promoting migration and retention of these cells in peripheral tissues[104]. Mouse cDC2s are considered a specialised subset promoting CD4+ T cell responses, and the same ability may be predicted for human cDC2s, based on their superior ability to express genes associated with MHCII processes[72]. Indeed, blood, skin and lung cDC2s are capable of inducing Th1, Th2 and Th17 polarization of CD4+ T cells, depending of the source of antigen and stimulation[47,92,105,106]. Moreover, lung cDC2s are more efficient than cDC1s in promoting Th17 response after *A. fumigatus* exposure[76], consistent with their ability to secrete IL23 and with the role of their murine counterparts in bacterial and fungal infections. However, human cDC1s and cDC2s in

blood, lymph nodes and lung appeared to be equally competent in inducing Th1 responses in the presence[91,107] or absence of specific activation stimuli[64,91], as well as promoting a Th17 response in the intestine[28]. Emerging evidence supports a role of human cDC2s in Th2 response in allergy and asthma, consistent with the mouse model[108–113]. However, mouse moDC have also been associated with allergic responses[108]. Therefore a better characterisation of the phenotype of cDC2s and monocyte-derived DCs in humans and their contribution to asthma and allergy is required. Finally, gene expression profiles and functional analysis support the idea that human cDC2s may be involved in the regulation of tissue homeostasis, consistently with their mouse CD11b+ counterparts. Human cDC2s in non-lymphoid tissues express high levels of immune regulatory molecules, such as CLEC4A (DCIR), ILT4, ICOS-L and PD-L1[28,114]. Moreover, human cDC2s have been shown to secrete IL-10 in response to *E. coli* or LPS stimulation, and to efficiently induce immunosuppressive T cell responses[115,116].

1.4.3 Human pDCs subset

In steady state human pDCs localise mainly in blood and lymphoid organs, whereas during inflammation, they can migrate and can be detected in non-lymphoid tissues as well[117,118]. pDCs are phenotypically defined as CD11c- HLA-DR+ CD123+ BDCA2/CD303+ BDCA4/CD304+ CD45RA+ cells[117,119], and they can be further subdivided, based on their expression of CD2, in $CD2^{lo}$ and $CD2^{hi}$ pDCs[120,121]. Moreover, a subset of pDCs expressing CD56 have been recently characterised[122], which also express CD33, CD13, CD2 and CD46 and it has been proposed to represent an immature form of pDCs [122]. Consistent with the mouse system, human pDC development relies on the transcription factors IRF8 and E2-2. Indeed, autosomal recessive mutation of IRF8 corresponds to the lack of pDCs in the affected subjects[71]

and impairment in pDC function has been reported in E2-2-deficient patients[117,123]. One of the most peculiar characteristics of pDCs is their ability to sense viral and self nucleic acids through the highly expressed Toll-like receptors 7 and 9 (TLR7 and TLR9)[124–126]. Once activated, pDCs can produce massive amounts of type I interferon (IFNα and IFNβ) and for this reason they are considered one of the most important effectors of antiviral immunity[117,120,127–130]. The ability of pDCs to secrete type III interferon further supports this concept[77,131]. Indeed, pDCs have been shown to play a role in the immure response against several pathogenic viruses, of which the most extensively characterised is HIV [132]. Likewise, the extremely efficient production of type I interferon by pDCs has been associated with non-viral pathogen immune responses. The contribution of human pDCs has been reported in *Aspergillus fumigatus* infection by producing type I interferon in a Dectin-2 dependent manner[133] as well in responses against BCG, where pDCs are considered a fundamental mediator of the optimal CD8+ T cell response to vaccine[134]. Moreover, pDCs contribute to the priming of CD4+ T cells by DC2s in mycobacterial infection[135], and their activation and consequent IFNa production has been described in human blood and tonsils in response to numerous gram positive and negative bacteria[136]. The involvement of pDCs in several autoimmune diseases has been reported primarily as the major source of IFNα and β[126]. An "IFN signature" has been described in systemic lupus erythematosus (SLE)[137–139], psoriasis[140], Wiskott-Aldrich syndrome (WAS)[141] and atherosclerotic lesions[142]. In all these pathologies, the high levels of type I IFN are mainly due to pDC activation in response to self nucleic acids via TLR7 and TLR9[137–142]. Finally, even if capable of initiating a powerful inflammatory response, pDCs have also been associated with tolerance and immunoregulation[143]. Based on experimental evidences and clinical observations, a potential role of pDC has been suggested in protection from allergy[144],

transplant tolerance[145], induction of regulatory T cells[146] and poor prognoses in patients with solid tumors[147].

1.4.3.1 Human pDC in T cell responses

Human pDCs are characterised by superior ability to stimulate naïve T cells as compare to their mouse counterparts[134]. Indeed, blood pDCs have been reported to efficiently induce Th1[127,143] and Th2[143] polarisation of CD4+ T cells. However, whether this is true for lymphoid organ pDCs has not yet been established. Moreover, experimental evidence demonstrates that pDCs are also capable of cross-presenting soluble[60,63,148–150], viral[150–152] and cell-associated[148,153] antigens. Nevertheless, the ability of human blood pDCs to prime T cells has been recently challenged. It has been demonstrated that the CD11c- CD123+ BDCA2/CD303+ BDCA4/CD304+ CD45RA+ cells originally used *in vitro* to assess T cell activation by pDCs, are actually a heterogeneous population containing a "contaminating" fraction of cells described either as DC precursors (pre-DC)[154] or as an alternative DC subset (AS DCs)[62]. These cells can be discriminated from pDCs based on the expression of CD33, Siglec6 and Axl[62,154]. More interestingly, when deprived of this additional population of cells, pDCs failed to efficiently induce T cell proliferation[62,154].

1.4.4 Human DCs in blood: additional subsets

The existence of additional subsets other than cDC1, cDC2 and pDC has been recently described in human blood[62]. The unbiased analysis of single-cell RNA sequencing data in HLA-DR+ lineage- cells from healthy donor PBMCs enabled the identification of ten independent populations within the blood HLA-DR+ lin- cells. Six of these clusters were described as dendritic cell subsets, based on their gene expression profile, phenotype and function, whereas the remaining four populations appeared to be monocytes. According to the original classification, the lin- HLA-DR+ fraction of

human PBMCs consists of three subsets of dendritic cells (cDC1, cDC2 and pDC, i.e.) and three subsets of monocytes (CD14+ "classical", CD16+ "non-classical" and CD14+ CD16+ "intermediate", i.e.). Therefore, three additional DC subsets, named as DC3, DC4 and AS DCs as well as one monocyte subset (mono 4) were revealed with this approach[62]. DC4s were described as poorly characterised CD11c+ CD1c- CD141- cells and no further information was provided about their function or phenotype, which potentially overlaps with previously described CD16+ SLAN+ "DC-like" monocytes, also named as SLAN DCs. DC3s represent a second subset of BDCA1/CD1c+ cells characterised by a more "inflammatory" signature of their gene expression profile, which can be distinguished from classical DC2s based on the lower expression of CD1c and CD11c. Finally, a new population of Axl+ Siglec6+ cells (AS DC) was identified. AS DCs shared expression of many genes and surface markers with pDCs, but functional characterisation of these cells confirmed their independence from the pDCs subset. Indeed, pDCs were uniquely capable of secreting high amounts of type I interferon in response to TLR9 stimulation, whereas AS DCs could produce IL12-p70 when stimulated with TLR4 and TLR7/8 agonists, suggesting a closer relationship with conventional DCs[62]. Moreover, AS DCs have been shown to be able to induce T cell proliferation as efficiently as the other cDCs subsets, while pDCs failed to do so when deprived from the AS DCs "contamination" due to their partially overlapping phenotype[62]. Based on this unbiased approach a new classification of the human blood DC compartment has been proposed[62]. However, the existence of these subsets in lymphoid and non-lymphoid organs and a further characterisation of their phenotype and function will require further investigation.

1.4.5 Human inflammatory DCs

Little is known about inflammatory dendritic cells (infDCs) in human, even though recent publications have reported the isolation of human infDCs displaying specific features consistent with their mouse counterparts[60,155]. Mouse infDCs have been described *in vivo* in a variety of inflammatory conditions[156–166]. Mouse infDCs are characterised by the expression of surface markers mostly shared with other myeloid populations, for instance inflammatory macrophages (infMacs), and therefore their isolation may sometimes present a challenge. However, infDC can be clearly distinguished from infMacs due to their unique ability to migrate to the lymph nodes in a CCR7-dependent fashion[108,156,167] and efficiently activate T cells[108,161]. Moreover, infDC have been described as monocyte-derived cells that can differentiate in the tissues during inflammation[156], and this concept is further supported by their dependency on CCR2[160,161,168–172]. InfDC are not affected in *Flt3l*$^{-/-}$ mice[108], suggesting their independence from the DC lineage. Although infDCs express the DC-lineage specific transcription factor ZBTB46 *in vitro*[173] and *in vivo*[174], they were poorly labelled in *Zbtb46*^{cre} *x Rosa*^{lslYFP} fate mapping experiments[50].

Based on the observation that infDC-like cells can be generated *in vitro* from mouse monocytes in the presence of GM-CSF[175]and the identification of infDC in several GM-CSF-dependent inflammatory settings[50,163,166], GM-CSF has been proposed to play an essential role in infDC development.

More recently, inflammatory cells with similar characteristics have been identified in human inflamed tissues in atopic dermatitis patients, arthritis synovial fluid and tumor ascites[60,155]. Human infDCs were characterised by the expression of CD14, CD1c, CD11b, CD172α, FcεRI, CD11c, HLA-DR, CD1a, CD206, M-CSFR/CD115 and ZBTB46[60,61,176]. Moreover, in the same inflammatory conditions, infDCs can be distinguished from infMacs based on their expression of CD1c and the lack of CD16[60].

Transcriptomic profiles of infDCs isolated from human inflamed tissues consistently align with GM-CSF-dependent monocyte-derived DCs generated *in vitro*, supporting the monocytic origin of these cells[60]. Little is known about the function of human infDCs *in vivo*. However, human infDCs isolated from synovial fluid of arthritis patients and from tumor ascites have been shown to induce Th17 polarisation of CD4+ T cells *ex vivo* through the secretion of the Th17-polarising cytokines TGFβ, IL1β, IL6 and IL23[60].

1.5 Growth factors involved in the MPS development

The development of monocytes, macrophages and dendritic cells is a strictly regulated process that involves essential growth factors specific for each lineage. Based on *in vitro* and *in vivo* evidences both in mouse and human, three fundamental cytokines can be identified: Flt3L, GM-CSF and M-CSF.

1.5.1 Flt3L

Flt3 ligand (Flt3L) is a key regulator of dendritic cell commitment during hematopoiesis[6,177] and it is ubiquitously secreted by a wide range of cells, including stromal cells, endothelial cells, activated T cells[178] and activated mast cells[179]. Flt3L is produced as a trans-membrane protein biologically active in both soluble and membrane-bound forms. Soluble Flt3L is the product of proteolytic cleavage of the extracellular domain of the trans-membrane protein[180,181].

The fms-like tyrosine kinase 3 (Flt3), also known as Flk2 or CD135, is the cognate receptor for Flt3L. Flt3 is expressed in all early hematopoietic progenitors both in human and mouse, including short-term repopulating hematopoietic stem cells (HSCs)[182–184], common lymphoid and myeloid progenitors (CLP and CMP, respectively)[185], monocyte and dendritic cell progenitor (MDP)[186,187], common dendritic cell progenitor (CDP)[6,7,45] and circulating DC precursors (per-DCs)[8,34]. Flt3 expression

is lost in all committed progenitors before entering the circulation and it is maintained exclusively in pre-DC and in the terminally differentiated DC subsets[8,185]. The crucial role of Flt3L in dendritic cells development has been fully demonstrated *in vivo* using both Flt3- and Flt3L-deficient mice, which display impaired DC development, without affecting monocyte numbers and function[8,17]. Interestingly, Flt3 knockout mice presented a less severe phenotype as compared to the ligand-deficient ones[17,186,188,189], suggesting that a potential alternative unknown receptor might be involved. Albeit limited in number, the few DCs still present in Flt3L-deficient mice appeared to be fully functional[17]. Moreover, the transfer of fully functional DCs into recipients lacking Flt3L resulted in reduced proliferation of the cells[186], potentially suggesting a more important role of Flt3L in DC homeostasis rather than development. In contrast with this hypothesis, Flt3L-deficient mice have been reported to display reduced numbers of CDPs[190] and pre-DCs[186]. Further studies have supported the fundamental role of Flt3L in dendritic cell development and maintenance. For instance, high levels of Flt3L have been detected in both mouse[188,191] and human[44] serum in response to Flt3 inhibition or cDC depletion, suggesting a strictly regulated feedback between DC availability and Flt3L concentration. Furthermore, the administration of Flt3L was reported to selectively expand DCs *in vivo* in mouse[17,186,192,193] and in human[34,98,99,194]. Likewise, Flt3L can successfully support DCs differentiation *in vitro* from human cord blood-derived progenitors[68,69] or mouse bone marrow cells[7].

Finally, although monocyte development is not affected *in vivo* in Flt3L-deficient mice, the addition of Flt3L to *in vitro* cultures of human monocytes in the presence of GM-CSF and IL4 increased the yield of DC-like cells and their ability to stimulate T cells proliferation[195]. Therefore, a potential effect of Flt3L in driving the differentiation of monocyte-derived cells cannot be excluded.

1.5.2 GM-CSF

Granulocyte macrophage colony-stimulating factor (GM-CSF or CSF-2) is a hematopoietic cytokine controlling myeloid cell development, the most prominent role of which is granulocyte and monocyte differentiation and maintenance[196]. GM-CSF binds specifically to its receptor GM-CSFR (CSF-2R), which consists of a trans-membrane protein formed by two subunits, GM-CSFRα and GM-CSFβ1. GM-CSFR expression has been reported in committed hematopoietic progenitors (GMP, MDP and CDP), as well as terminally differentiated cells, such as monocytes, cDC1s and cDC2s[158,197].

GM-CSF, alone or in combination with other factors, has been extensively used to induce *in vitro* differentiation of DC-like cells from either monocytes or hematopoietic progenitors, both in human[33,198] and mouse[199]. Indeed, GM-CSF still represents a key factor in the generation of DC-based vaccines against cancer using irradiated tumor cells, a procedure termed as GVAX[200,201]. However, even if numerous publications have demonstrated that *in vitro* cultures with GM-CSF give rise to moDC that do not resemble the physiologically circulating DC subsets[60,202], GM-CSF is still part of the cytokine mix most commonly used to differentiate human DCs *in vitro* from hematopoietic progenitors[34,45,62,154,203,204].

In vivo, GM-CSF appeared to be mainly involved in the homeostasis and differentiation of myeloid cells in peripheral tissues, especially during inflammation. Indeed, conventional DCs are not affected in the lymphoid organs of mice lacking either GM-CSF or GM-CSFR expression[205]. Conversely, a reduction of cDC1 and cDC2 in the lung, intestine and dermis has been reported in GM-CSF-deficient mice[158,197,206], suggesting a critical role for GM-CSF in cDC maintenance in non-lymphoid tissues[158]. Importantly, GM-CSF has been also identified as a key regulator of CD103 expression in tissue cDCs[158,207–209].

1.5.3 M-CSF

Macrophage colony-stimulating factor (M-CSF or CSF-1) is a hematopoietic cytokine involved in the regulation of development, proliferation and survival of monocytes and macrophages[210]. Alternative splicing of the M-CSF mRNA gives rise to two forms of the translated protein with different fate: a soluble form that is incorporated into vesicles and released via the secretory pathways, and a trans-membrane form which can play a role in cell-cell interactions or alternatively be released into the extracellular space by proteolytic cleavage[211,212].

M-CSF receptor (M-CSFR, CSF-1R or CD115) is a member of the class 3 receptors tyrosine kinases (RTK-III) and is expressed in hematopoietic progenitors at different stages of myeloid commitment, including GMP and MDP, whereas it is lost in CDP and pre-DCs[8,45]. M-CSFR is also expressed in terminally differentiated cells such as macrophages, monocytes and a subset of mouse cDC2s[190,213]. Therefore, based on the pattern of expression of their receptors in monocyte and dendritic cell progenitors, Flt3L and M-CSF have been proposed as key mediators in the commitment of MDP towards the monocyte or dendritic cell fate[178]. Moreover, an alternative ligand for M-CSFR has been identified in interleukin 34 (IL-34)[214].

M-CSF involvement in macrophages and monocyte development is strongly supported by *in vivo* evidence in the mouse model. Mice lacking M-CSFR display normal DC development in lymphoid organs, but they failed to develop monocytes, macrophages and Langerhans cells (LCs)[215]. On the other hand, when the production of the ligand is impaired (i.e., in *Csf-1*$^{-/-}$ *or Csf-1*$^{op/op}$), monocytes and macrophages are still absent, but LCs develop normally in the epidermis[216]. This observation suggested the existence of a second ligand for M-CSFR, which has been identified in IL-34[214]. Indeed, *Il34*$^{-/-}$ mice

exclusively lack epidermal LCs, whereas dermal DCs and macrophages are not affected[158,217].

In vitro evidence further supports the role of M-CSF in macrophages development. Indeed, M-CSF alone or in combination with other factors can be used to efficiently generate monocyte-derived macrophages from hematopoietic progenitors both in humans and in mice[32,218].

1.6 Transcriptional regulation of DC development

The transcriptional regulation of dendritic cell development has been extensively investigated in mice[93,219,220]. Genetic evidence demonstrated the key role of subset-specific transcription factors in the differentiation, maintenance and function of cDC1s, cDC2s and pDCs (Figure 1-2).

Conventional DC1 (CD8α+/CD103+) development strictly depends on the expression of the interferon regulatory factor 8 (IRF8)[221–224], the basic leucine zipper transcription factor ATF-like 3 (BATF3)[90,208] and the inhibitor of DNA binding protein 2 (ID2)[225]. Mouse cDC2 instead, are depleted in the absence of interferon regulatory factor 4 (IRF4) expression[92,100,226–228] and they also rely on interferon regulatory factor 2 (IRF2)[229], RELB[230], RBP-J[231], NOTCH2[21] and Kruppel-like factor 4 (KLF4)[22]. Moreover, KLF4 and NOTCH2 dependency discriminate between two subsets of cDC2 that can be further characterised by the expression of the endothelial cell-selective adhesion molecule (ESAM)[21]. Both cDC1 and cDC2 specifically express the zinc finger transcription factor ZBTB46, a conserved feature between mouse[173,232] and human[47,60]. However, mouse ZBTB46 is not required for cDC development and appears to be involved in the maintenance of a DC specific transcriptional program and in the suppression of DC activation[173,232]. Finally, E2-2 (TCF4) has been identified as a master regulator of pDC development both in human and in mouse[123], and it can in turn control

the activation of other transcription factors involved in pDC development (SPIB, IRF8) and function (IRF7)[123].

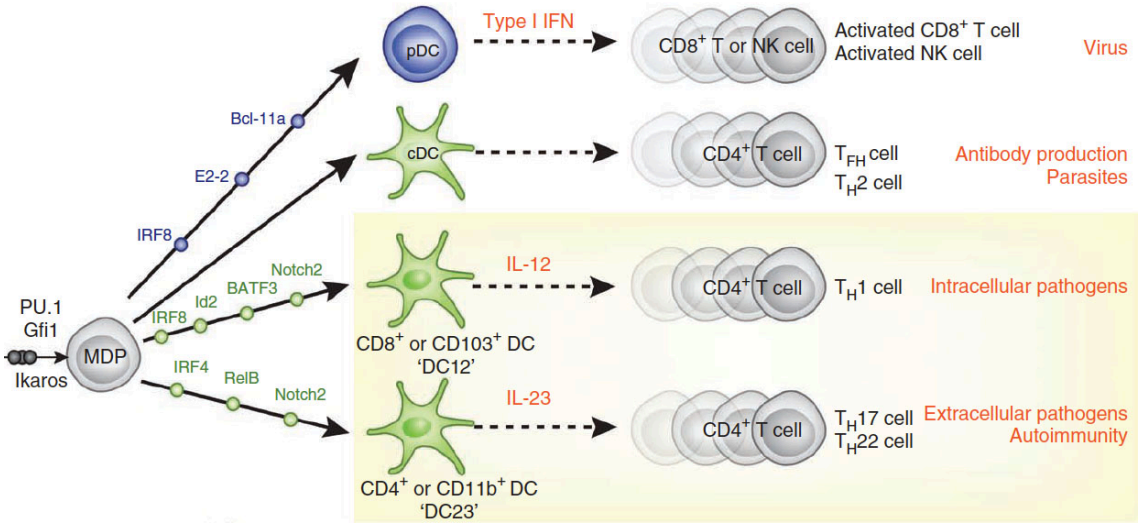


Figure 1-2. Transcriptional regulation of mouse DC subsets (adapted from Satpathy et al, 2012[219]). Transcription factors sequentially required for mouse cDC and pDC development. Subset specification is further defined by their ability to respond differently to pathogen challenges and produce subset-specific cytokines involved in the polarization of naïve CD4+ T cell.

1.6.1 IRF8

In mouse, IRF8 has been clearly identified as a key transcription factor involved in cDC1 and pDC development. Two distinct phenotypes have been reported in mice carrying dysfunctional IRF8. The *Irf8*$^{-/-}$ mice display a more severe phenotype characterised by the absence of monocytes, pDCs and cDC1s, while impairing the functional maturation of cDC2s[221,222]. A second model providing insightful information about IRF8 function in dendritic cell development is the BXH2 mouse. The BXH2 mouse harbours a spontaneous point mutation in the *Irf8* gene (IRF8^{R294C}), which impairs exclusively the development of cDC1 subset without affecting pDCs and monocytes[223]. These mice exhibit an immunodeficient phenotype similar to the *Irf8*$^{-/-}$ animals, characterised by enhance susceptibility to *Mycobacterium Bovis* (BCG), splenomegaly, increased granulocyte count and development of spontaneous myeloid

leukaemia[233]. More recently, the involvement of IRF8 in different stages of monocytes and dendritic cell development has been assessed in several conditional *Irf8*$^{-/-}$ mice[224]. This analysis enabled the identification of IRF8 as a key transcription factor involved in both cDC1 development and maintenance (terminal selector), whereas its absence in terminally differentiated pDCs exclusively affects their function but not their survival. Moreover, the deletion of IRF8 in the bone marrow progenitors highlighted a fundamental role of this transcription factor in the differentiation of CDPs from MDPs as well as the development of monocytes from their cMoP precursors[224]. IRF8 expression requires prior autoactivation of IRF8, which is dependent on different transcription factors at different stages of DC development. Indeed, it has been demonstrated that while IRF8 expression in CDP and pre-DC1 is sustained by its PU1-dependent autoactivation, the terminal differentiation of these progenitors into DC1 requires a BATF3-dependent autoactivation of IRF8[234]. This model is supported by *in vivo* evidences in *Batf3*$^{-/-}$ mice, which displayed an impaired development of terminally differentiated DC1 without affecting the development of CDP and pre-DC1[234].

In humans, the understanding of the transcriptional regulation of DC development relies on the identification of specific mutation associated with DC deficiencies. To date, the DC deficiency syndromes include well-characterised mutations of *IRF8* and *GATA2*. Two different mutations on the *IRF8* gene have been reported in patients affected by DC deficiency syndrome: the autosomal recessive mutation K108E and the autosomal dominant sporadic mutation T80A[71], both localised in the DNA-binding domain of IRF8. The K108E mutation appeared to recapitulate the absence of functional IRF8 in both the *Bxh2*$^{-/-}$ and *Irf8*$^{-/-}$ mouse models, and it is characterised by the increased susceptibility to *Mycobacterium* and other bacteria/virus infections and the appearance of a striking myeloproliferative syndrome[71]. This very severe phenotype is explained by the complete lack of cDC1s, cDC2s, pDCs and monocytes in the peripheral blood of

patients harbouring the K108E mutation. Conversely, patients affected by the autosomal dominant sporadic mutation T80A display normal monocytes, pDC and cDC subsets in the blood. However, within the cDC compartment a specific loss of CD1c+ DC2s has been described, while the CD141+ DC1s were not affected[71]. This represented a completely unexpected observation considering the higher expression of IRF8 in cDC1s and pDCs and its essential role in their development and maintenance in the mouse model, suggesting a poorly conserved regulation of DC development between human and mouse. Moreover, patients harbouring the T80A mutation presented a less severe phenotype, displaying an increased susceptibility to mycobacterial infection but normal life expectancy and no need for stem cell transplantation therapy[71].

1.6.2 GATA2

The DC, monocyte, B and NK lymphoid (DCML) deficiency syndrome is another group of genetic disorders, which provide further evidence of the regulation of DC development in human. DCML was described as 'autosomal dominant and sporadic monocytopenia'[235] and is characterised by heterozygous mutations in the transcription factor *GATA2*[236,237]. Many different mutations have been identified, all resulting in the loss or mutation of the C-terminal zinc finger domain[236]. *GATA2* mutations are considered the cause of dendritic cell, monocyte, B and natural killer lymphoid deficiency and correlate with increased susceptibility to infection, leukemic transformation and pulmonary alveolar proteinosis, higher incidence of solid tumors and in some cases autoimmunity[238]. Patients harbouring GATA2 mutations completely lack circulating monocytes, cDCs and pDCs and the remaining fraction of HLA-DR+ Lin- cells were identified as CD34+ progenitors. The lack of circulating B cells, NK cells, monocytes and DCs reflects the absence of multilymphoid progenitors (MLP) and granulocyte-macrophage progenitors (GMP) in the bone marrow[44], and it is associated

with increased levels of Flt3L in the serum as well as depletion of regulatory T cells (Treg)[44]. Similar observations have been reported in conditional *GATA2* knockout mice, which are characterised by significant reduction of the DC population[239]. GATA2-deficient progenitors displayed a reduction in myeloid-related gene expression along with an increase in T lymphocyte-related genes, suggesting an important role of GATA2 in the progenitor commitment to the myeloid rather than lymphoid lineage[239].

1.6.3 E2-2

The basic helix-loop-helix transcription factor E2-2 (TCF4) is a member of the E proteins family and it has been identified as a key transcriptional regulator of human and mouse pDCs[123]. Constitutive or inducible knockout of E2-2 completely abrogates pDCs development in mice. Moreover, haploinsufficiency of E2-2 led to impaired type I interferon responses as well as aberrant gene expression profiles in pDCs both in mouse and in the human Pitt-Hopkins syndrome[123]. The pivotal role of E2-2 in pDCs regulation is further supported by its ability to modulate the activation of other transcription factors involved in pDC function and differentiation, such as *IRF7*, *IRF8* and *SPIB*. Interestingly, E2-2 has been reported to repress the expression of ID2, a transcription factor involved in conventional DC1 development. Conversely, ID2 is capable of inhibiting E2-2 function, suggesting that a finely regulated balance between these two factors is crucial to determine the plasmacytoid versus conventional DC fate[117,240].

1.6.4 ID2

Genetic experiments in the mouse model enabled the identification of the helix-loop-helix transcription factor ID2 as an essential factor driving mouse conventional DC1 development. Indeed, ID2 is highly expressed during mouse DCs differentiation *in vitro*

and *Id2*$^{-/-}$ animals completely lack splenic cDC1s as well as Langerhans cells[225]. The role of ID2 in human DCs development has not been evaluated yet and the consequences of its abrogation are still unknown. However, ID2 has been identified as a key regulator of hematopoietic progenitors commitment and its overexpression in human cord blood-derived progenitors resulted in the expansion of the HSC pool as well as the modulation of progenitors commitment towards the erythromyeloid lineage, at expense of lymphoid differentiation[241].

1.6.5 IRF4

The fundamental role of the transcription factor IRF4 in cDC2 development and maintenance has been extensively investigated in mouse[92,100,226,227]. *Irf4*$^{-/-}$ mice display severe reduction of mouse cDC2s in the spleen[100], as well as in peripheral tissues including lung, gut and intestine[92,226], with the consequent impairment of both Th2 and Th17 responses[92,226,227]. Furthermore, *Irf4*$^{-/-}$ bone marrow progenitors failed to differentiate *in vitro* in cDC2[100]. However, even if high expression of IRF4 in human DC2s has been reported[92], its role in their differentiation and maintenance has not been formally proven yet.

Table 1-2. TF mutations associated to DCs deficiency in human and mouse

TF	Species	Mutation	Mono	cDC1	cDC2	pDC	Ref
IRF8	*Mus musculus*	*Irf8*$^{-/-}$	absent reduced	absent	impaired function	absent	221 222 224
		Bxh2	normal	absent	normal	normal	223
	Homo sapiens	K108E	absent	absent	absent	absent	71
		T80A	normal	normal	abnormal phenotype	normal	
GATA2	*Mus musculus*	*Gata2*$^{-/-}$	reduced	reduced	reduced	reduced	239
		Gata2$^{+/-}$	normal	normal	normal	normal	
	Homo sapiens	*GATA2*$^{+/-}$	absent	absent	absent	absent	235 236
E2-2	*Mus musculus*	*E2-2*$^{-/-}$	normal	normal	normal	absent	123
		E2-2$^{+/-}$	normal	normal	normal	impaired	

TF	Species	Mutation	Mono	cDC1	cDC2	pDC	Ref
						function	
	Homo sapiens	*E2-2*$^{+/-}$	normal	normal	normal	impaired function	123
Id2	*Mus musculus*	*Id2*$^{-/-}$	normal	absent	normal	normal	225
	Homo sapiens	n/a	unknown	unknown	unknown	unknown	-
IRF4	*Mus musculus*	*Irf4*$^{-/-}$	normal	normal	reduced	normal	92 226
	Homo sapiens	n/a	unknown	unknown	unknown	unknown	-

1.7 Ontogeny of human dendritic cells

Both mouse and human DCs in the peripheral tissues are constantly replenished by specific precursors residing in the bone marrow. In humans, clinical observations support this notion, as demonstrated by the complete replacement of dermal DCs after bone marrow transplantation[47], as well as the total absence of circulating DCs in patients undergoing bone marrow suppression in pre-engraftment stage of bone marrow transplantation[42].

The ontogeny of monocytes and DCs has been originally described in the mouse model based on the identification of clonogenic progenitors with restricted potential to differentiate into monocytes, DCs or both. In the mouse bone marrow, a monocyte and dendritic cell progenitor (MDP)[187] has the ability to differentiate into common dendritic cell progenitors (CDP)[6–8], which has lost monocyte and macrophage potential, and common monocyte progenitor (cMOP)[242]. CDP can give rise exclusively to plasmacytoid DC and precursors of conventional DCs (pre-DC)[163], which egress the bone marrow and through circulation, seed the peripheral tissues where they give rise to both cDC1 and cDC2. DC potential has been described in human CD34+ progenitor compartment both in multi-lymphoid progenitors (MLP) and in the granulocyte and macrophage progenitors (GMP)[243–247]. More recently, the human counterparts of

MDP[45], cMoP[46], CDP[45] and pre-DC[34,62,154] have been identified in human cord blood and bone marrow. Moreover, precursors committed exclusively to either the cDC1 or cDC2 fate have been described both in mice[248] and humans[154,204] (Figure 1-3). Nevertheless, the phenotype of the human circulating pre-DC precursors, including the already committed pre-DC1s and pre-DC2s, has not reached a full consensus. Breton et al. first identified human pre-DCs in blood, bone marrow and peripheral lymphoid organs as lin- CD34- CD45RA+ CD135+ CD116+ CD123int CD117+[34], and described the existence of pre-committed progenitors for cDC1s (CD172a-) and cDC2s (CD172a+) based on the expression of CD172a[204]. More recently, single-cell mRNA sequencing and cytometry by time-of-flight (CyTOF) analysis on human PBMCs were used to identify human DC progenitors[154]. Pre-DCs were described as lin- CD34- CD45RA+ CD135+ CD123+ CD33+, a phenotype similar to the one described previously[34]. However, few differences were highlighted such as the higher levels of CD123 expression, the inclusion of the CD117- cells as pre-DCs and the need to assess CD33 expression to distinguish them from pDC (CD33-)[154]. Moreover, even if pre-DC1s and pre-DC2s were defined as CD1c- CADM1+ (pre-DC1) and CD1c+ CADM1- (pre-DC2) within the CD123lo/- fraction, the pattern of expression of CD172a was consistent with the previously reported phenotype[204]. Finally, a more divergent phenotype for DC progenitors was identified by unbiased transcriptomic analysis of blood PMBCs[62]. CD45RA+ CD34int CD100+ cells were shown to give rise to all DCs subsets, including pDCs, which suggests a less committed state of these precursors more consistent with CDPs. However, these cells did not express the cytokine receptors CD123, CD135, CD115 and CD116 previously reported either in MDP, CDP[45] or pre-DCs[34,154]. Therefore, it is not clear if these cells might represent a less committed precursor, as suggested by the authors and supported by their more primitive

morphology and phenotype[62]. Nevertheless, further studies are needed to clarify whether these phenotypically different precursors are related and how.

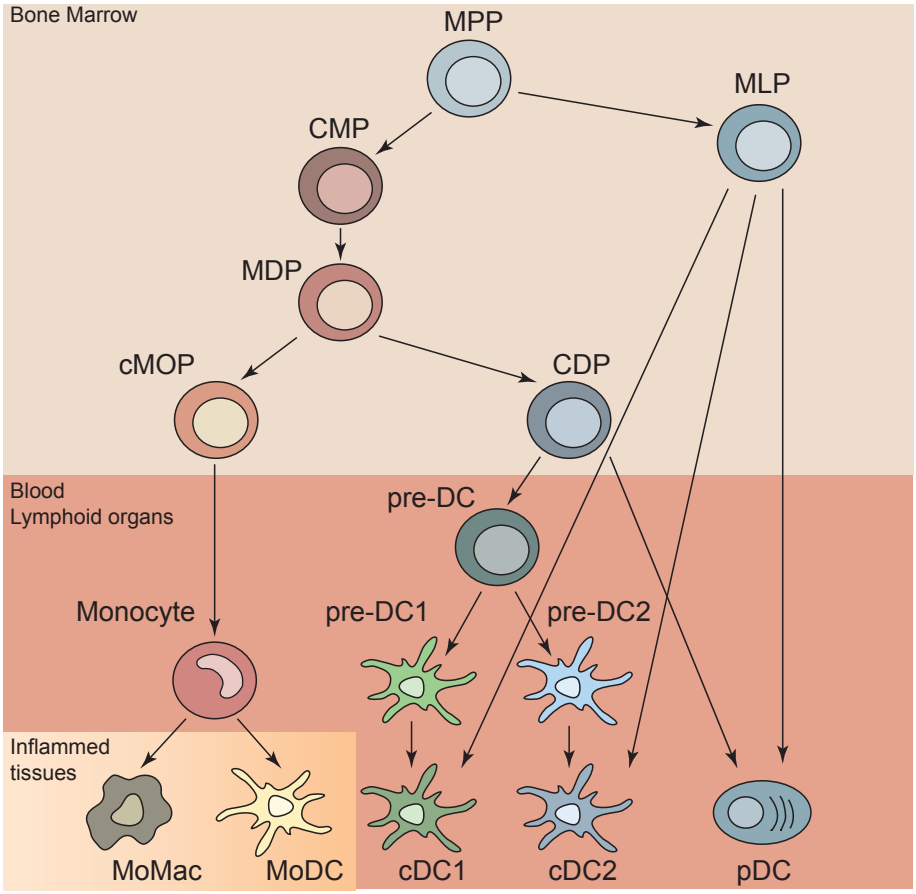


Figure 1-3. Ontogeny of human mononuclear phagocyte system. Human DCs and monocytes arise from a common progenitor, the monocyte and dendritic cell progenitor (MDP) residing in the bone marrow. MDPs give rise to both monocyte- and DC-committed progenitors, the cMOP and CDP, respectively. CDPs differentiate into circulating pre-DC that can be further subdivided in subset-specific precursors, the pre-DC1 and pre-DC2, which can give rise to terminally differentiated DC1 and DC2, respectively. Moreover, DC potential has been recently described in early hematopoietic progenitors, suggesting that DCs can also originate from human multipotent lymphoid progenitors (MLP)[247].

1.8 The bone marrow niche

Adult hematopoietic stem and progenitor cells are localised in the bone marrow. Since its colonisation by hematopoietic stem cells migrating from the fetal liver during embryonic development, the bone marrow represents the primary site of hematopoietic development throughout adult life. In the bone marrow, HSC and hematopoietic progenitors reside in defined regions supporting their maintenance and expansion: the hematopoietic stem cell niche. The concept of a hematopoietic niche in the bone

marrow, and its importance for HSC function, was originally postulated in the late 1970s by Ray Schofield, based on the superior ability of the bone marrow to support HSCs as compare to the spleen[249]. The endosteal surface of the bone was initially considered the principal component of the niche, suggesting a regulatory role for the osteoblastic lineage in hematopoietic maintenance[250–252]. More recently, technical improvements in the live imaging techniques along with the identification of more specific markers to visualise HSCs, enabled the localisation of these cells in proximity of the sinusoidal vasculature[253], revealing a primary role of perivascular cells in the HSC regulation[254–257]. The conditional knockout of essential niche factors in perivascular cells and osteoblasts further supported this hypothesis[258–260]. However, several cell types have been described as important components of the hematopoietic niche[261] (Figure 1-4).

1.8.1 Cellular composition of the niche

Perivascular cells have been proposed to be the main regulators of HSC maintenance and regeneration in the bone marrow niche. In human, this population of cells has been characterised as CD146+ stromal progenitors[262], and a fraction of them also express platelet-derived growth factor receptor-α (PDGFRα), CD51 (ITGAV) and nestin[263,264], defining them as bone marrow mesenchymal stem and progenitor cells (MSPCs)[263]. Perivascular stromal cells comprise at least four overlapping and highly redundant cells types that can be defined as CXCL12-abundant reticular (CAR) cells[265], cells marked by the expression of nestin (Nes-GFP^{dim} and Nes-GFP^{bright})[264], leptin receptor-expressing cells (LepR)[259] and cells expressing the transcription factor osterix (Osx-Cre)[258]. All these populations are the major producers of essential niche factors, such as CXCL12 and stem cell factor (SCF). Conditional deletion of CXCL12 in all subsets showed an increased mobilisation of HSCs, whereas the abrogation of SCF production resulted in

their complete depletion[258–260,266], confirming an essential role of perivascular stromal cells in the regulation of the HSC maintenance.

Early *in vitro* studies suggested that osteoblasts could sustain HSCs self-renewal and differentiation[267]. However, mouse genetic experiments have recently demonstrated that osteoblasts do not contribute to HSCs maintenance by producing key factors of the niche[258–260] and they do not associate with hematopoietic progenitors[254,255]. Endothelial cells (ECs) have been reported as a further component of the bone marrow niche. ECs isolated from bone marrow can support expansion and differentiation of human CD34+ hematopoietic progenitors *in vitro*[268], and the abrogation of angiogenic activity of ECs impaired their ability to support long-term HSCs[269]. More importantly, the selective deletion of either CXCL12 or SCF in ECs resulted in decreased numbers of HSCs in the bone marrow, highlighting the important contribution of these cells for a functional niche[259,260]. Moreover, other cell types including members of the sympathetic nervous system[270–273], macrophages[274,275], megakaryocytes[276,277] and adipocytes[278,279] have been proposed to actively influence HSCs homeostasis, by directly or indirectly modulating the niche microenvironment.

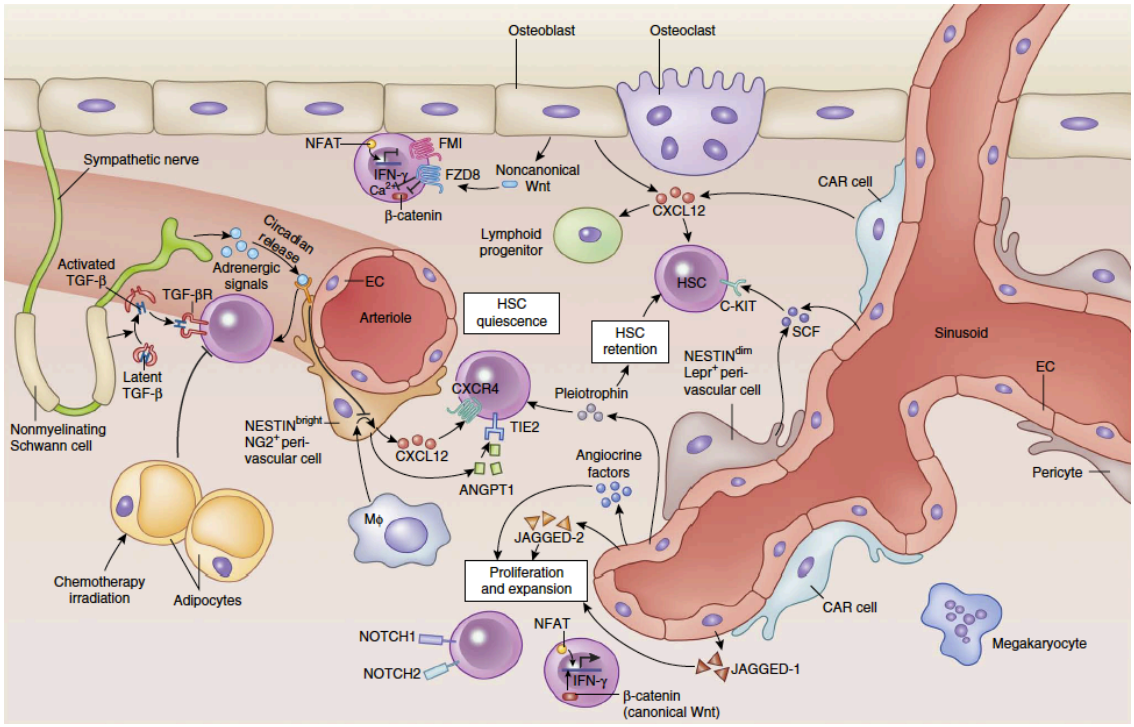


Figure 1-4. Key cell types regulating HSC maintenance in the niche (Mendelson et al, 2014[280]). Schematic of the cell types constituting the bone marrow niche. Key growth factors regulating HSC maintenance and expansion (e.g. CXCL12 and SCF) are produced by perivascular stromal cells residing in the bone marrow, which can be subdivided in four main cell types: CAR cells, Nestin^{dim} Lepr^{+} cells, Nestin^{bright} cells and cells expressing the transcription factor osterix (Osx-Cre cells).

1.8.2 Growth factors of the niche

HSC quiescence, self-renewal and differentiation in the bone marrow niche are finely regulated processes that require a balanced contribution of many factors provided by the surrounding microenvironment. Genetic experiments in the mouse model allowed the identification of essential factors involved in HSC maintenance and mobilization such as CXCL12 (SDF1), SCF and Thrombopoietin (TPO). However, other important signalling pathways have been associated with HSC regulation and function, even if their actual contribution has not been fully elucidated and their implication is still controversial.

Table 1-3. Essential growth factors of the bone marrow niche

Growth factor	Mouse model	Localisation/target	HSCs	Ref.
CXCL12 (SDF1)	*Cxcl12*$^{-/-}$	Global/CXCL12	Absent	
	Cxcr4$^{-/-}$	Depleted	Absent	
	LepR-Cre X Cxcl12$^{fl/fl}$	Perivascular/CXCL12	Increased mobilisation	259
	Osx-Cre X Cxcl12$^{fl/fl}$	Perivascular/CXCL12	Increased mobilisation	258 286
	Tie2-Cre X Cxcl12$^{fl/fl}$	Endothelial cells/CXCL12	Reduced numbers	
	Col2.3-Cre xCxcl12$^{fl/fl}$	Osteoblast/CXCL12	Not affected	
	Vav-Cre X Cxcl12$^{fl/fl}$	Hematopoietic cells/CXCL12	Not affected	
SCF	*c-Kit*$^{-/-}$	Global/c-kit	Impaired repopulation	287
	Sl/Sl^{d}	Global/membrane-bond SCF	Absent	288
TPO	*Mpl*$^{-/-}$	Global/Mpl	Reduced numbers	295
	TPO$^{-/-}$	Global/TPO	Reduced numbers	293

1.8.2.1 CXCL12

The C-X-C motif chemokine 12 (CXCL12), also known as stromal cell-derived factor (SDF1) is a key chemokine involved in HSC maintenance and more importantly in their retention in the bone marrow niche[265,281–284]. Perivascular stromal cells (CAR, LepR-Cre, Nes-GFP, Osx-Cre) represent the main source of CXLC12 in the bone marrow, by expressing 100 to 1000-fold higher levels than endothelial cells and osteoblasts, respectively[258,260,265,285,286]. The critical role of CXCL12 in HSC homing and maintenance has been demonstrated in several mouse models. The global deletion of CXCL12, as well as its receptor CXCR4, resulted in the depletion of HSCs from BM[265,281,287]. Moreover, conditional knockouts of CXCL12 in perivascular stromal subsets (LepR-Cre and Osx-Cre) displayed increased mobilization of HSCs from BM [258,260], while its deletion restricted to endothelial cells (Tie2-Cre) appeared to reduceHSC numbers without affecting their retention[258,260]. Conversely, the contribution

of CXCL12 produced by other bone marrow tissues is dispensable for HSC maintenance, as demonstrated by its conditional ablation in osteoblasts (Col2.3-Cre and Sp7-Cre) or hematopoietic cells (Vav1-Cre)[258,260] (Table 1-3).

1.8.2.2 SCF

Stem cell factor (SCF) is a cytokine expressed by a number of cells types that interact with its receptor c-Kit, a class III tyrosine kinase receptor expressed on hematopietic stem and progenitor cells. SCF can be produced in both its soluble or membrane-bond forms, depending on alternative mRNA splicing and proteolytic cleavage. SCF acts on HSCs by promoting their survival[288], and its fundamental role in the niche microenvironment has been extensively demonstrated in mouse genetic experiments (Table 1-3). HSCs lacking c-Kit expression showed an impaired repopulation potential[288]. On the other hand, HSCs are absent in mice exclusively expressing the soluble form of SCF (*Sl/Sl*^{d}), highlighting the essential role of membrane-bond SCF for HSC maintenance[289,290]. This was further demonstrated by the preferential localisation of HSCs in proximity of trans-membrane SCF-expressing cells in a niche containing both wild-type and *Sl/Sl*^{d} stromal cells[281]. Moreover, membrane-bound SCF plays a role in the retention of HSCs in the niche. Indeed the proteolytic cleavage of SCF mediated by the receptor activator of nuclear factor NF-kB (RANK) ligand and cathepsin K produced by osteoclasts, increases mobilisation of HSCs in the niche[291]. Since cell-cell contact is required for a functional SCF signalling, SCF expression has been used to identify the most important cell components of the niche.

1.8.2.3 TPO

Thrombopoietin (TPO) is a cytokine primarily involved in megakaryocyte and platelet development, even if an important role of TPO and its receptor Mpl in the maintenance of hematopoietic progenitors has been described[292–295]. Indeed, mice lacking the expression of both TPO and Mpl display decreased numbers of repopulating HSCs in

the bone marrow[296] (Table 1-3). Furthermore, TPO has been shown to contribute to the generation and expansion of definitive HSCs in the developing embryo, and to promote HSC survival *in vitro*[297,298]. Liver and kidney represent the main source of TPO *in vivo*, even though low levels of expression were also detected in the bone marrow stroma[299,300]. However, the conditional knockout of TPO in niche stromal cells is needed to assess the importance of their contribution.

1.8.2.4 Other factors

Other factors have been implicated in the regulation of HSC homeostasis in the bone marrow niche, even if the experimental demonstration of their involvement is still a source of controversy.

Notch signalling was initially proposed as an important pathway for HSC regulation in the bone marrow niche[301], and this was supported by the observations that Notch is capable to expand HSCs *ex vivo* in gain-of-function experiments. Moreover, Notch activity was detected in transplanted HSPCs[302] and Notch ligands were detected in the endosteal surface and associated with increased HSC activity[250]. However, *in vivo* knockout models failed to confirm these observations[303,304].

Wnt/β-catenin represents a very complex signalling pathway, whose involvement in the hematopoietic niche has not been fully elucidated. Early studies suggested that HSC maintenance in the bone marrow is not affected by the absence of Wnt signalling[305,306]. However, later studies revealed the osteoblast-specific expression of important mediators of the pathway, such as dikkopf homolog 1 (DKK1), secreted frizzled-related protein 1 (SFRP1) and Wnt inhibitor factor 1 (WIF) and reported a consistent effect of their modulation on HSC self-renewal and exhaustion[307,308]. Based on these observations, Wnt appeared to be involved in HSC maintenance by promoting proliferation and long-term repopulation activity.

1.9 DCs and cancer immunotherapy

The series of events characterising the immune response against cancer have been recently summarised in the concept of "Cancer-Immunity Cycle" by Chen and Mellman[309], and in this context dendritic cells play a major role in the initiation and maintenance of an antitumor immune response (Figure 1-5). The Cancer-Immunity Cycle is defined by seven major steps leading to the establishment of a self propagating immune response and ending with the killing of cancer cells. The process starts with the release of cancer cell antigens (step1) that can be engulfed and transported to the lymph nodes by antigen presenting cells (APCs) (step 2). Here, APCs efficiently prime and activate antigen-specific cytotoxic T cells (CTLs) (step 3), which migrate through circulation (step 4) and infiltrate the tumor (step 5). Once at the tumor site, CTLs can recognise (step 6) and kill the cancer cells[309].

Dendritic cells play a fundamental role in this process by capturing and processing tumor-associated antigens (TAA) and presenting them to T cells. In order to induce an efficient cytotoxic T cell response, antigen phagocytosis is followed by DC maturation. Matured DCs reduce their phagocytic ability, express co-stimulatory molecules and migrate in a CCR7-dependent fashion toward the lymph nodes, where they are capable of secreting key cytokines to efficiently promote T cells activation[310–314]. For this reason, in the last two decades several DC-based strategies have been proposed for the therapeutic vaccination against cancer, including (i) non-targeted protein- and nucleic acid-based vaccines that can be captured by DCs *in vivo*, (ii) peptides/proteins coupled to antibodies targeting DCs specifically and (iii) *ex vivo* generated dendritic cells loaded with tumor antigens[310]. Two main approaches have been tested so far as a source of *ex vivo* generated DCs: the *in vitro* differentiation of DC-like cells from monocytes (moDCs) and the isolation, expansion, activation and re-injection of naturally circulating subsets. Since their first discovery[33], moDCs have been considered a

promising source of clinically relevant cells, mainly due to the high numbers of monocytes that can be easily isolated from blood and differentiated into moDCs by the exposure to GM-CSF and IL-4. For this reason, numerous clinical trials have been performed in the last twenty years, and a quite extensive knowledge of the efficacy of this approach is now available[310,315]. Unfortunately, although moDC-based vaccination appeared to be safe and to successfully induce the expansion of circulating tumor-specific CD4+ and CD8+ T cells, they failed to demonstrate a long-lasting beneficial effect on the overall clinical responses[315,316]. A better understanding of the mechanisms by which DCs interact with T cells and contribute to establish an efficient immune response highlighted important aspects that can explain the disappointing outcomes of moDC-based vaccination. Indeed, many limitations of moDC-based therapy have been identified, such as the poor migration to lymph nodes, the expression of inhibitory signals (IL-10, PD-L1 and PD-L2, e.g.) and an inefficient antigen loading and presentation[314,317–320]. For this reason, more recently the paradigm of DC-based vaccination shifted towards the use of naturally circulating subsets[315]. Indeed, preliminary results on patients affected by metastatic melanoma and treated with *ex vivo* expanded, activated and antigen-loaded pDCs, demonstrated that despite the limited magnitude of a melanoma-specific immune response, the pDC-based therapy resulted in a consistent increase of the patients overall survival[321]. Supporting this concept, a cell-based therapy consisting in the *ex vivo* activation and antigen loading of isolated antigen-presenting leukocytes, showed a significant increase in the overall patients survival[322], and received FDA approval for the treatment of prostatic cancer in 2010 (Sipuleucel-T/Provenge®). These clinical studies contributed to the understanding that tumor growth is closely associated to one or more dysfunctional steps of the cancer-immunity cycle, preventing a durable anti-tumor response. Several aspects may concur to the cycle deregulation, including insufficient antigen expression, dendritic cell

paucity and immaturity, subversion of DC function by tumors, lack of T cell infiltration and inhibition of T cell effector functions. Therefore, therapeutic approaches that aim at re-establishing a functional cycle may represent a successful strategy to induce tumor regression. Supporting this hypothesis, a recent publication demonstrated how an efficient expansion and activation of DCs in the tumor site along with PD-L1 blockade therapy can successfully improve the immune response against mouse melanoma and protect the animals from secondary tumors[323].

In this regard, the use of genetically modified iPSC-derived DCs may represent a valuable alternative to overcome some of the previously described limitations by providing an efficient platform to produce patient-specific DCs that (i) resemble the physiologically circulating subsets, (ii) can be *in vitro* manipulated to constitutively express tumor-associated antigens, (iii) can be *in vitro* activated by TLR stimulation to promote immunogenic responses and efficient T cell priming, (iv) can be genetically modified to abrogate signalling pathways negatively regulating different phases of the cancer-immunity cycle, such as antigen presentation (IL-10, IL-13 or IL-4, e.g) or T cell priming and activation (PD-L1 and PD-L2, e.g.). The PD-1/PD-L1 pathway represents a very promising candidate for such approach.

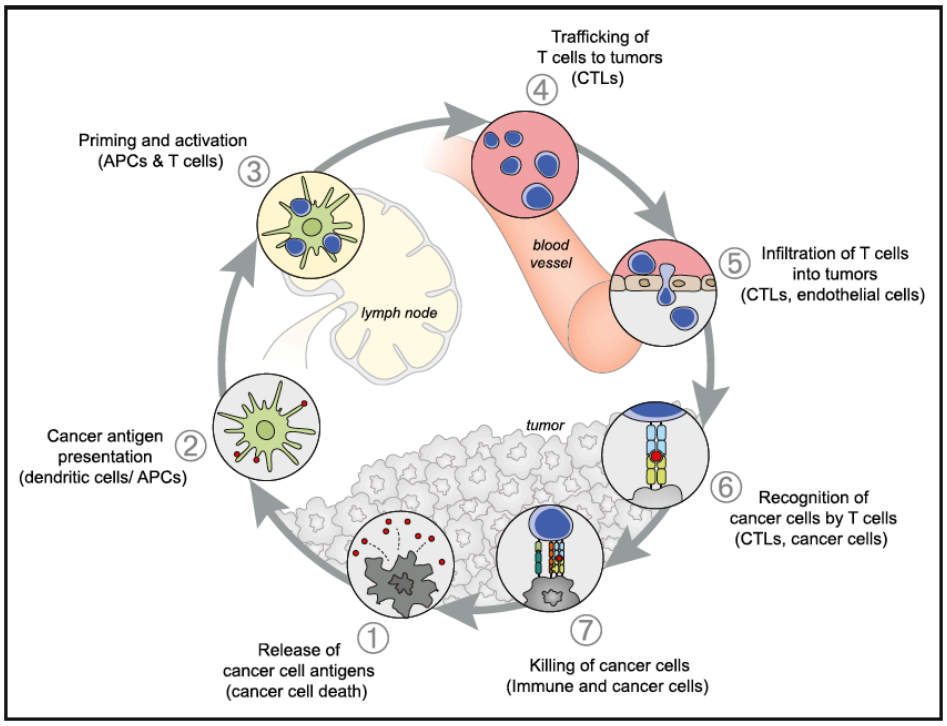


Figure 1-5. The Cancer-Immunity Cycle (Cheng and Mellman, 2013[309]). The Cancer-Immunity Cycle represents the series of events characterising the immune response against cancer. Antigen presenting cells, including DCs, pick up cancer cell antigens in the tumour site (1) and migrate towards the lymph nodes (2) where they are capable of presenting tumour-associated antigens (TAA) to T cells (3). Activated T cells can then migrate through the circulation (4) and infiltrate into the tumour (5), where they recognise and kill cancer cells (6-7).

1.9.1 PD-1/PD-L1 axes

Programed death 1 (PD-1) is an immune-checkpoint receptor known to negatively regulate T cell activity. When engaged by its specific ligands PD-L1 and PD-L2, PD-1 induces apoptosis of the effector cell and therefore promotes tolerance by limiting T cell activity[324–328]. The expression of PD-L1 and PD-L2 can be induced in dendritic cells and macrophages as well as in activated B and T lymphocytes. Despite its predominant role in suppressing the immune system to prevent autoimmune diseases, PD-1 plays an essential role in responses against cancer [329,330]. Indeed, many types of malignant cells express its ligands and can therefore prevent tumor eradication by inhibiting cytotoxic T cells activity[329,331]. Therefore, the PD-1/PD-L1 pathway represents one of the most successful targets of the immune checkpoint blockade in cancer therapy[332–334]. In this regard, numerous antibodies that abrogate the PD-1/PD-L1 axes have been developed in

pre-clinical studies, targeting both the receptor and the ligand (Table 1-4). Noteworthy, two anti-PD-1 antibodies (Nivolumab and Pembrolizumab) have been lately approved by the FDA to treat metastatic melanoma, non-small cell lung cancer (NSCLC), head and neck squamous carcinoma (HNSCC) and Hodgkin lymphoma[335–340]. Furthermore, three anti-PD-L1 antibodies (Atezolizumab, Durvalumab, Avelumab) were recently approved for the treatment of bladder cancer[341]. Moreover, a recent publication has shown that the anti-PD-L1-mediated immunity in mouse melanoma is strictly dependent on the presence of dendritic cells, suggesting a major involvement of the PD-1/PD-L1 pathway in the interaction of T cells with DCs in the lymph nodes, rather than with cancer cells in the tumor site[323]. For all these reasons, targeting PD-L1 in iPSCs may represent a potential strategy to increase the immunogenicity of iPSC-derived DCs against cancer.

Table 1-4. PD-1 and PD-L1 antibodies in clinical development

Agent	Target	Status
Nivolumab (MDX1106, BMS-936558)	PD-1	FDA approved for NSCLC, Hodgkin lymphoma, renal cell carcinoma, melanoma
Pembrolizumab (MK-3475)	PD-1	FDA approved for NSCLN, H&N, melanoma
Pidilizumab (CT-011)	PD-1	Phase I/II clinical trial ongoing
BMS-936559 (MDX-1105)	PD-L1	Phase I clinical trial completed
Atezolizumab (MPDL3280A)	PD-L1	FDA approved bladder cancer
Durvalumab (MEDI4736)	PD-L1	FDA approved bladder cancer
Avelumab (MSB0010718C)	PD-L1	FDA approved Merkel-cell carcinoma
AMP-224	PD-L2 fusion	Phase I clinical trial completed

1.10 Modelling human DCs development

Human circulating DCs represent a very rare population of cells accounting for approximately 1% of blood PBMCs. For this reason, the use of *ex vivo* isolated DC subsets to better understand human DC function, regulation and development has proven to be a very challenging task. Therefore, the development of *in vitro* and *in vivo* systems to faithfully recapitulate DC differentiation from hematopoietic progenitors represents a fundamental tool for the study of human DC biology. For instance, the establishment of an *in vitro* platform supporting human DC differentiation enabled the identification of human MDP, CDP and pre-DC[34,336] as well as the characterisation of human BATF3-dependent Clec9A+ cDC1 cells, equivalent to the mouse cDC1 subset[66,68]. Moreover, the *in vitro* generation of DC-like cells (moDCs) from easily accessible human blood monocytes[33], has provided an extensively used and invaluable platform to study human antigen presenting cells (APCs) and their interactions with T lymphocytes.

Based on their unique ability to engulf, process and present antigens to T cells[15], DCs represent an essential component of therapeutic vaccination against cancer. Therefore, the idea of developing reliable approaches to obtain patient specific DCs in order to induce a strong and specific response against cancers has gained increasingly interest in the last decades. However, the absence of a significant clinical benefit of the moDC-based therapy[316] and the very limited number of circulating DCs preventing their extensive use in anticancer immunotherapy, represent important drawbacks of this kind of approaches.

In conclusion, the need to further elucidate the mechanisms underlying human DC biology as well as their potential clinical application in cancer immunotherapy, highlight the importance of establishing a reliable system to faithfully recapitulate human DC development *in vitro*.

1.10.1 Modelling human DC development *in vitro*

Many attempts to generate human DC subsets *in vitro* have been performed in recent years, based on the use of growth factors known to be important in DC maintenance and differentiation (Table 1-5).

The first experimental evidence that human monocytes can be induced to differentiate into DC-like cells upon exposure to GM-CSF and IL-4 *in vitro*, was provided by Sallusto and Lanzavecchia in 1994[33]. This culture system represented an important technical advancement, enabling the study of key aspects of human APC functions *in vitro*. However, it is now commonly accepted that moDCs do not represent a faithful model of the naturally circulating subsets, due to important differences on their phenotype and function[50,202].

Pioneering work from Caux et al. also demonstrated the feasibility of differentiate human Langerhans cells and moDCs from cord blood-derived CD34+ hematopoietic stem and progenitor cells (HSPCs), paving the way for the development of more refined approaches to induce cDC and pDC differentiation form hematopoietic progenitors[198,342,343].

More recently, several methods have been reported to support the differentiation of human DC subsets from HSPCs (Table 1-5). All these approaches were based on the use of recombinant growth factors involved in HSPC maintenance and expansion, as well as DC differentiation. In some of the protocols mouse stromal feeder lines were used[34,203,344], whereas others provided an extra step of HSPC expansion[68,202]. More importantly, all these methods were based on the use of GM-CSF, a cytokine dispensable for DC development *in vivo*[205] and know to induce moDC differentiation both *in vitro*[33] and *in vivo*[50]. Based on their phenotype, subset-specific TLR response/cytokine production and gene expression profiles, the generation of cDC1s,

cDC2s and pDCs were reported. pDCs were characterised by the expression of CD123+, CD303+ and CD45RA+ in most of the protocols, and they were further validated by their ability to produce massive amounts of type I interferon in response to TLR7 and TRL9 stimulation[34,203,344]. cDC1s were also identified based on their specific expression of Clec9A (DNGR1), even if some discrepancies in their phenotype were observed when compared to blood DC1s, including the lack of CD141 expression[69] or the presence of markers expressed in tissue DC1s such as CD1c, CD1a and CD172a[34,68,69,202,203]. Nevertheless, gene expression profiles of *in vitro* generated cDC1s aligned them to their human blood counterparts[34,202,203]. Finally, the differentiation of cDC2s was described in most of the protocols[34,69,203]. However, an extensive evaluation of the observed heterogeneity (CD14 expression, e.g.) was not performed[34,203] and the potential contribution of monocyte-derived cells to the CD1c+ subset was not assessed. Indeed, when compared to *in vitro* generated moDCs, the gene expression profile of XCR1- CD1c+ DC2-like cells was consistent with their monocytic origin[202].

Table 1-5. Culture systems supporting human DCs differentiation *in vitro*

Input	Feeder	Growth factors		Output	Ref.
		Step I	Step II		
Mono	-	GM-CSF IL-4		moDC	310
CD34+ HSPCs	-	GM-CSF TNFa SCF	GM-CSF TNFa	$CD14^{-}CD1a^{+}$ LC $CD14^{+}CD1a^{+}$ moDC	198 341
CD34+ HSPCs	OP9	Flt3L IL-7		$CD123^{+}CD303^{+}$ pDC	344
CD34+ HSPCs	-	Flt3L SCF IL-3 IL-6	Flt3L SCF GM-CSF IL-4	$CD141^{+}Clec9A^{+}CD1c^{+}CD1a^{+}$ DC1	66
CD34+ HSPCs	-	Flt3L TPO		$CD141^{-}Clec9A^{+}CD172^{int}$ DC1 $CD14^{-}CD1c^{+}CD172a^{+}CD11b^{+}$ DC2 $CD123^{+}CD172a^{int}$ pDC	69

CD34+ HSPCs	-	Flt3L SCF IL-3 TPO	Flt3L SCF GM-CSF IL-4	$CD141^+XCR1^+Clec9A^+CD1c^+CD1a^+$ DC1 $CD1c^+CD1a^+CD206^+CD11b^+XCR1^-$ moDC	202
CD34+ HSPCs	MS5	Flt3L SCF GM-CSF		$CD141^+Clec9A+CD1c^+$ DC1 $CD1c^+CD172a^+CD14^{+/-}$ DC2 $CD123^+CD303^+CD45RA^+$ pDC	203 204

1.10.2 Modelling human DCs development *in vivo*

The generation of mouse models supporting DC differentiation *in vivo* represents an essential tool for the study human DC development and functions in a more physiological environment, allowing the evaluation of their interactions with other cell compartments. In this regard, the generation of humanised mice models enabled a wide range of possibilities for the study of human immune system. Indeed, humanised mice have been extensively used as a model to study human hematopoietic hierarchy, human malignancies (mostly hematopoietic), and to perform preclinical studies on newly generated therapeutic approaches[345]. The "humanisation" process is achieved by transplantation of human hematopoietic progenitors into recipient mice. The successful engraftment relies on some fundamental features of the hosting animal: i) the absence of innate and adaptive immune responses, ii) the availability of a niche to favour human progenitors persistence and expansion and iii) the presence of cross-reacting signals supporting human cells maintenance and differentiation.

Several mouse strains permissive to human hematopoietic cells engraftment have been generated in the last 30 years[346]. The abrogation of the mouse immune system has been achieved by targeting the main effectors of the innate and adaptive responses, including B and T lymphocytes, NK cells and macrophage phagocytosis. The absence of mouse B and T cells is a consequence of the defective V(D)J recombination, which occurs naturally in mice harbouring the severed combined immunodeficiency (SCID) (as a consequence of the spontaneous autosomal recessive mutation *Prkdc*^{scid})[347–351], or in

alternative can be induced by targeting one of the recombination activating genes RAG1 or RAG2[352–356]. Moreover, deficiency of NK cells can be achieved by mutation of the *Il2rγ* gene, coding for the common gamma chain of cytokine receptors, which is share among different member of the IL-2 family, including IL-15 and resulting in the complete abrogation of NK cells development[354,355,357–359]. Furthermore, phagocytic tolerance against human cells can be induced by triggering the CD47-SIRPα pathway, also known as "don't eat me" signal. SIRPα is trans-membrane protein expressed in phagocytic cells, whose interaction with the endogenous CD47 results in phagocytosis inhibition. In most mouse strains, mouse SIRPα does not bind to human CD47 thus mediating phagocytosis of human cells. In mouse models permissive to human hematopoietic cells, this interaction is achieved either by introducing the human *SIRPA* gene as a bacteria artificial chromosome (BAC) or by replacing its mouse homologous (knock-in)[360,361], or by generating the humanized mouse in the non-obese diabetic (NOD) background, whose *Sirpa* allele is very similar to the human one and interacts with human CD47[362]. The combination of these different strategies allowed the generation of numerous humanised mice models over the years, which are listed in Table 1-6.

Table 1-6. Humanized mice models

Strain	Name	Features	Ref.
CB17-*Scid*	SCID	T and B cells deficiency	346, 347
NOD-*Scid*		T and B cells deficiency phagocytic tolerance	348, 349, 350
NOD-Scid *Il2rg*$^{-/-}$	NSG	T, B and NK cells deficiency phagocytic tolerance	356, 357, 358
BALB/c-*Rag2*$^{-/-}$ *Il2rg*$^{-/-}$	BRG	T, B and NK cells deficiency	354
Tg(hSIRPA)*Rag2*$^{-/-}$ *Il2rg*$^{-/-}$	SRG	T, B and NK cells deficiency phagocytic tolerance	360

A second requirement for a successful engraftment is the availability of a hematopoietic niche accommodating the transplanted human progenitors, and this has been generally

achieved by sub-lethal irradiation of the recipient mice. However, alternative approaches have been reported recently, based on generation of genetically modified animals characterised by impaired HSC maintenance, which confers a competitive advantage to human progenitors homing in the bone marrow niche[363–365]. Furthermore, the development of a human immune system in humanized mice is strictly dependent on external growth factor provided by the surrounding environment. Therefore, human cells maintenance and differentiation mainly relies on the cross-reactivity of mouse cytokines with human receptors, which only occurs for a limited number of factors[346]. The lack of sufficient cytokine stimulation is responsible for the developmental and functional defects that still characterised the human hematopoietic cells generated in humanised mice[346,366,367]. This includes the limited differentiation of functional myeloid and NK cells[368,369], the lack of B cells maturation[370,371] and the impaired selection and maturation of human T cells[372,373]. To overcome this issue, a new approach has been developed, based on the generation of transgenic animals expressing human hematopoietic factors either by replacing their mouse counterparts or under the control of a constitutively active promoter. This strategy has been used to generated humanised models expressing human M-CSF[374], membrane-bond SCF[375,376], TPO[365], GM-CSF and IL-3[377,378] and the combination of M-CSF, IL-3, TPO and GM-CSF[379]. The most representative example of this cohort of models is the generation of the MISTRG mouse[379]. Indeed, the combinatorial expression of human M-CSF, IL-3, TPO and GM-CSF resulted in the ability of these mice to develop a functional myeloid compartment, capable of triggering an effective innate response against viruses and bacteria[379]. However, even if the full characterisation of human DC subsets has not been reported for this model, the observed myeloid compartment mainly comprised CD14+ and CD14+ CD16+ monocytes and macrophages, suggesting that the additional

effect of Flt3L may be required to induce an efficient DC differentiation. In fact, successful generation of all human DC subsets has been described in humanised mice treated with human Flt3L[70,179] (Table 1-7), providing experimental evidences that functional DCs can be generated *in vivo* from human hematopoietic progenitors.

Table 1-7. Differentiation of human DC subsets in humanized mice

Strain	Treatment	Irradiation	DC subsets	Time	Ref.
NOD-*Scid*	-	Yes	$CD11c^+$ $HLA\text{-}DR^+$ cDC $CD303^+HLA\text{-}DR^+$ pDC	8-10 weeks	[380]
NSG	DNA encoding huFlt3L	Yes	n/a	12 weeks	[381]
NSG	-	Yes	$CD141^+Clec9A^+$ DC1 $CD303^+CD123^+$ pDC	8-24 weeks	66
NOD-*Rag1*$^{-/-}$ *Il2rg*$^{-/-}$	huFlt3L	Yes	$CD141^+$ DC1 $CD1c^+$ DC2 $CD303^+$ pDC	8-10 weeks	179
NOD-*Scid*	huFlt3L	Yes	$CD141^+Clec9A^+$ DC1 $CD1c^+$DC2 $CD303^+CD123^+$ pDC	4-5 weeks	70

2 Hematopoietic system during embryonic development

Embryonic hematopoiesis represents a very organised and finely regulated developmental program, extremely conserved among the different animal models used to study early embryo development, such as zebrafish, frog, chick and mouse[382,383]. Hematopoietic cells originate from mesoderm, one of the three embryonic germ layers from which muscle and connective tissues are also generated. This process is strictly regulated and organised in space and time, and it occurs both in extra- and intra-embryonic sites: the yolk sac and the embryo proper[384]. The first wave of hematopoietic cells with limited potential originates from the yolk sac and support the early phases of embryo development[385]. Afterwards, the first hematopoietic stem cell (HSC) showing self-renewal capability and multi-lineage potential is detected in the aorta-gonad-mesonephros (AGM) region of the mouse embryo proper[386]. HSCs then migrate to the fetal liver where they undergo maturation and expansion before seeding the bone marrow, where they reside in a specialised niche to support the hematopoietic system during adulthood. The spatio-temporal organisation of embryonic hematopoiesis is a conserved feature of human and mouse embryonic development, even if the duration of each step is considerably different. In mouse, the subsequent stages occurring during embryogenesis can last less than a day, and are defined by specific embryonic days (E). On the contrary, in humans these intervals last significantly longer, and a different classification, known as Carnegie stages (CS), is used to describe the external morphological changes during embryo development[387]. The fact that embryonic hematopoiesis takes place in different sites led to the idea of a primitive program taking place in the yolk sac, as opposed to a definitive program occurring in the intra-embryonic tissues. More recently this oversimplified view has been challenged and the identification of further programs (EMPs, LMPPs, i.e.) with “definitive” features occurring in the yolk sac is a good example of the complexity in deciphering the actual

separation of these two processes. Moreover, the temporal organisation of hematopoietic cell generation in the embryo led to the concept of an embryonic hematopoiesis organised in waves, which consist in the sequential activation of distinct programs in very defined intervals of time.

2.1 Primitive versus Definitive Hematopoiesis.

The terms “primitive” and “definitive” hematopoiesis have been used in a rather broad sense, and this may sometimes represent a source of confusion in the interpretation of the available literature. In this regard, Medvinsky et al. pointed out that the term “definitive” hematopoiesis refers to all hematopoietic cells originating from a definitive HSCs (dHSCs), historically defined as a stem cell that can give rise to all the mature blood lineages in the adult hematopoietic system[388]. However, the authors acknowledge that the term definitive hematopoietic cells has been used to define cells that can give rise to myeloid and adult enucleated erythroid cells, in a less accurate way[388].

On the other hand, Lacaud and Kouskoff underlined that primitive hematopoiesis is most commonly defined as the first wave of hematopoietic cells that takes place in the yolk sac and generates primitive erythrocytes, megakaryocytes and macrophages[389–392]. Conversely, definitive hematopoiesis includes all the alternative hematopoietic programs occurring in the embryo after the primitive one, including Erythromyeloid progenitors (EMPs) in the yolk sac, Lymphoid-primed multipotent progenitors (LMPPs) in the yolk sac and para-aorta splanchnopleura (P-Sp) and definitive HSC (dHSC)[389]. The relationship between these two programs is not fully elucidated, but the more accepted model supports the idea that embryonic (primitive) and adult (definitive) hematopoiesis are independently generated. Embryonic hematopoiesis serves to maintain the embryo until the definitive program arises through the specification of dHSCs that sustain life-long hematopoiesis. Therefore, a dual origin of hematopoietic

development can be hypothesised, where the yolk sac and AGM are considered sites for the primitive and definitive programs, respectively. Observations from non-mammalian vertebrates support this view[384,393,394]. An alternative hypothesis relies on the existence of a common ancestor (not detected in experimental assays) that gives rise to sequential waves of hematopoiesis: a first wave generating primitive hematopoietic cells (but no dHSC) and a second one where dHSCs are generates and mature in the AGM. On these premises, the yolk sac appears to be the only tissue where the hematopoietic system originates, and AGM represent an intermediate "educational" site where HSC develop prior to colonisation of the fetal liver[395].

2.2 Waves of Hematopoiesis.

In human and mouse embryo, hematopoietic cells originate in subsequent waves characterised by their time of appearance, localisation and potential.

2.2.1 Primitive hematopoiesis

The primitive hematopoietic program occurs in the yolk sac in a very precise stage of embryo development, corresponding to E7.5 in mouse and CS 7-8 in human[385,396]. Progenitors generated during primitive hematopoietic program originate from blast colony-forming cells (BL-CFC) through an intermediate precursor expressing endothelial markers[397–399] and show a very restricted potential, giving rise exclusively to embryonic erythrocytes, megakaryocytes and macrophages[385,391,400]. Embryonic erythrocytes can be easily distinguished from their adult counterparts by their larger size, nuclear retention (eventually lost)[385,396] and the expression of specific isoforms of globin. In human, primitive erythrocytes express ε-globin, whereas definitive erythrocytes express either γ-globin before birth or β-globin after birth[401]. Likewise, mouse erythrocytes express εy- and βH1-globin if generated during primitive

hematopoiesis and β-globin if they originate from the definitive program[402]. Conversely, embryonic megakaryocytes and macrophages are substantially indistinguishable from their definitive counterparts, even if they present some peculiar features, such as reduced ploidy and platelets production for megakaryocytes[391,400] and the non-monocytic origin of primitive macrophages[403,404].

2.2.2 EMP hematopoiesis

A second wave of hematopoietic cells arises in the mouse yolk sac at E8.25. These cells are distinct from the primitive program but they appear before the establishment of circulation and the identification of the first definitive HSC[385,405]. Based on their restricted potential to differentiate exclusively into erythroid, megakaryocytic and myeloid lineages, these cells were named as erythro-myeloid progenitors (EMPs) and they originate from endothelium via endothelial-to-hematopoietic transition (EHT)[406,407]. Originally identified as the onset of definitive hematopoiesis in the yolk sac[385,408,409], EMPs are now considered as a separate hematopoietic program distinct from primitive and definitive hematopoiesis. The most relevant experimental evidence supporting this notion is the ability to separate EMPs from both primitive and definitive progenitors based on the expression of cKit, CD41 and CD16/32 and the lack of SCA1 in the mouse embryo[410,411]. When cultured *in vitro*, EMPs isolated from mouse embryo have the potential to differentiated into erythrocytes, megakaryocytes, macrophages, neutrophils, eosinophils and basophils. More importantly, no lymphoid potential has ever been reported for these cells. *In vivo* transplantation experiments have shown that EMPs provide only a short-term erythroid, myeloid and platelets engraftment when compared to HSCs. Moreover, erythrocytes originating from EMPs can be distinguished from the definitive ones by their simultaneous expression of low levels of embryonic βH1-globin as well as adult β-globin[409]. In human, an EMP wave similar to the one

observed in mouse embryogenesis occurs at CS13-15, and even if it has not been formally proved yet, early studies support this observation[412].

2.2.3 LMPP hematopoiesis

Concurrently to EMPs appearance, B and T cells can be observed in the yolk sac and para-aortic splanchnopleura (P-Sp) before the establishment of circulation and the generation of the first dHSC[413–415]. Lymphoid-primed multipotent progenitors (LMPPs) have been identified in the yolk sac at E9-9.5[416]. LMPPs originate from Ve-Cadherin+ Tie2+ CD41- endothelial cells[413,415] and are characterised by their potential to generate T lymphocytes (both αβ and γδ)[413,414], B lymphocytes (type B1 and marginal zone)[413,417] and myeloid cells[416], partially overlapping the EMP hematopoietic program. In the yolk sac, EMP and LMPP programs together give rise to a great variety of hematopoietic lineages comparable to the HSC-derived cells in the definitive hematopoiesis. However, the existence of a common progenitor linking these two programs has not been reported, and the ontogeny of EMPs and LMPPs requires further investigation to elucidate the underlying mechanisms regulating these pre-HSC programs[418]. The main function of EMPs and LMPPs is to actively support embryo early life during development. However, there are several examples of cells in the adult that have an EMP- or LMPP-origin, suggesting that these embryonic-derived cells fulfil functions other than supporting early embryo development. Of note, the EMP-origin of most of tissue resident macrophages has been extensively demonstrated, even though some controversy still remains on the major path of ontogeny followed by the development of microglia in the brain[36,37,41,419,420]. Furthermore, type1 B cells in mouse peritoneum and γδ T cells identified in several epithelia derive entirely from embryonic LMPPs[413,414,421,422].

2.2.4 Definitive hematopoiesis and dHSCs

Definitive hematopoietic stem cells (dHSCs) can be detected in the AGM, vitelline and umbilical arteries from E10.5 in mouse[386,423] and CS13 in human[424–427]. Definitive hematopoiesis takes place following the primitive program in the embryo proper and ultimately gives rise to definitive hematopoietic stem cells (dHSCs). The transfer of quail embryos to chicken yolk sac provided the first experimental evidence supporting the intra-embryonic origin of dHSC[384,428]. Afterwards, the P-Sp/AGM region of the mouse embryo has been identified as the major site of dHSC generation in vertebrates, including human[386,426,427,429,430]. However, alternative locations have been described as sites of dHSC differentiation such as yolk sac vessels[407,423,431–433], vitelline and umbilical arteries[434,435], placenta[436], head vasculature[437], and endocardium[438]. It has been clearly established that dHSCs originate from the endothelial layer of the dorsal aorta[424,439,440] and this is supported by lineage tracing experiments in mice, using a Ve-Cadherin-specific cre-mediated fate mapping approach[441–444], and in human ESC-based *in vitro* evidences[399,445]. Hematopoietic cells bud off from the endothelium and enter the circulation. This specialised endothelium with hematopoietic potential has been named Hemogenic Endothelium (HE), and the transformation of flat aortic endothelium to hematopoietic cells (endothelial-to-hematopoietic transition or EHT) has been characterised in chicken embryos[440], mouse embryos[441] and observed in live microscopy[444,446,447].

The self-renewing HSC are localised in intra aortic hematopoietic clusters (IAHC) along with non self-renewing progenitors. In mouse, IAHC are localised in the ventral floor of the dorsal aorta and correlates with the presence of functional HSCs[448]. Similarly, humans IAHC are located in the ventral AGM region and the presence of functional human dHSC within the IAHC has been confirmed by immunophenotype analysis and *in vivo* repopulation studies in NSG mice[449]. Both in mice and in humans,

the number of cells in the IAHC of the AGM region largely exceeds the number of HSCs[426], which supports the idea that developing not-yet-transplantable HSCs with immature phenotype constitute part of the clusters[450–452].

2.2.4.1 Definitive HSCs phenotype and maturation

Definitive hematopoietic stem cells (dHSCs) are defined by their ability to give rise to all blood lineages (multipotency) as well as to daughter HSCs (self-renewal). In mice, HSCs were first identified by the ability of bone marrow cells isolated from a donor mouse to undergo clonal expansion in the spleen of an irradiated recipient[453,454]. For this reason, the progenitor originating the clone (dHSC) was called colony-forming-unit-spleen (CFU-S). The observation that single CFU-S can give rise to both differentiated cells and new CFU-S progenitors established the self-renewability behaviour of these multipotent progenitors[455,456].

Substantial efforts have focused on the identification of the precise phenotype of dHSCs both in humans and mice. During embryonic hematopoiesis the first dHSC originates in the AGM region of the embryo and these newly generated dHSCs can be identified as CD150- VE-Cadherin+ CD45+ CD117+ CD34+ CD41+/- cells in mouse[408,457,458], and VE-Cadherin+ CD45+ CD117+ CD34+ CD90+ CD105+ CD38- CD45RA- cells in human[449].

The ability of dHSC to engraft and provide long-term reconstitution (LTR) in lethally irradiated recipients represents an extremely powerful assay to evaluate the differentiation and maturation potential of newly generated dHSC. In mice, the first dHSC capable of repopulating an adult recipient appears in the AGM at E10.5[386,423,426,457]. Similarly, cells isolated from human AGM at CS 14 can successfully provide multi-lineage long-term engraftment in immunodeficient mice (NSG)[426]. However, both AGM and Yolk Sac-derived cells isolated at E9 can successfully engraft

neonatal recipients[459,460], suggesting that dHSCs precursors are already available at E9 in these sites, even if a further maturation step is required to acquire repopulation activity. Further evidences supporting this notion were provided by *in vitro* co-cultures of VE-Cadherin+ cells isolated from AGM at E9.5-10 on OP9 or endothelial feeder cells. After co-culture AGM-derived cells, named as pre-HSCs, acquired the ability to engraft adult recipient mice, even if yolk sac-derived cells isolated at the same stage of development failed to do so[450,451,461,462]. These experimental evidences led to a more precise classification of the maturation steps that dHSCs precursors undergo before acquiring a fully functional phenotype. In a three-step process, VE-cadherin+ CD45- pro-HSC first differentiate into VE-cadherin+ CD45- CD41+ type1 pre-HSC followed by the acquisition of CD45 expression and the consequent appearance of the VE-cadherin+ CD45+ CD41+ type2 pre-HSC which further mature in fully functional HSC with LTR capability[451,461]. Finally, the acquisition of MHCI expression marks the appearance of a fully functional matured dHSCs, characterised by LTR activity in conventional recipients[463]. An important role in promoting pre-HSC maturation is played by endothelial cells, a key component of the hematopoietic niche. Indeed, overexpression of adenoviral protein E4ORF1 in endothelial cells activates the AKT pathway promoting survival and inducing a state that closely resemble the endothelial cells in the hematopoietic niche[464]. AKT-activated endothelial cells have been shown to support maintenance and expansion of HSCs isolated from mouse AGM by inducing the expression of Notch ligands[462], as well as supporting the generation of engraftable HSCs from monkey iPSCs[465]. Moreover, extracellular matrices also play an important role in the hematopoietic niche. Sub-aortic mesenchyme underneath the IAHC produces extracellular matrix rich in Tenascin C[466]. Tenascin C is produced by overgrown OP9 *in vitro*, which appeared to be superior in promoting hematopoietic differentiation. Furthermore, Tenascin C promotes hematoendothelial differentiation of human iPSCs in

chemically defined conditions, and supports the generation of progenitors with multilineage potential but limited self-renewal[467].

2.3 Spatiotemporal progression of embryonic hematopoiesis

2.3.1 The yolk sac

The first appearance of hematopoietic cells in the embryo occurs in the yolk sac (YS) around E7.5 as a consequence of extra-embryonic mesoderm ingress through the posterior primitive streak and the subsequent differentiation into hematopoietic cells[468]. In the yolk sac, hematopoietic as well as endothelial progenitors are localised in the YS blood islands[469].

Based on the observation that the yolk sac is the site where the first hematopoietic cells appear, it was initially proposed that HSCs originate from YS progenitors. Supporting this hypothesis, the injection of donor cells isolated from the YS into embryonic circulation of a recipient mouse resulted in the detection of donor T cells in the recipient thymus[470]. However, experiments performed in the avian model and in Xenopus led to the opposite conclusion. In fact, the generation of quail-chicken and congenic chicken chimeras clearly showed that YS hematopoietic cells only transiently contribute to the hematopoietic compartment and the definitive hematopoietic program has an intra-embryonic origin[384,471]. Likewise, experimental evidence in *Xenopus laevis* established that YS and intra-embryonic blood compartments are independently generated and originate from different blastomers[393]. Moreover, additional evidence in the mouse model support this idea. Yolk sac cells at E8 (pre-circulation) are restrained to produce short-lived myeloid cells[472] and they lack CFU-S before E9.5[427] and dHSCs earlier than E11.5[386,429]. Only at E11.5 true dHSCs are detected in the mouse YS, which acquires the ability to expand them starting from E12.5[473]. Based on these observations it is reasonable to conclude that yolk sac hematopoiesis takes place in the absence of

dHSCs[388]. In addition, even though it is impossible to entirely exclude the ability of the YS to independently generate true multipotent dHSCs, their presence in the later stages of embryo development might represent the consequence of tissue repopulation by dHSCs of exogenous origin. The anatomical organisation of the human yolk sac significantly differs from the mouse. The mouse YS consists of a whole structure that surrounds the embryo, whereas in human it is a balloon-like formation of mesoderm origin, which is localised in front of the embryo and later differentiates into endothelial and hematopoietic erythroblast to support embryo formation. However, like in mouse, the human yolk sac is the first site of hematopoietic cell formation. At CS7-8 large embryonic erythrocytes arise, along with rare primitive macrophages and megakaryocytes[396,474]. Subsequently, at CS 10 the first erythroblast can be observed in the cardiac cavity followed by the appearance of the first CD45+ cells[425], underling the establishment of circulation, a further aspect shared with the mouse developing embryo[475].

2.3.2 The AGM

In the mouse embryo, the first CFU-S is identified in the AGM at E9.5 in higher numbers than in the yolk sac[427]. Also, the AGM has been described as a powerful source of dHSC activity[386] and AGM-derived cells are capable of long-term reconstitution as early as E10[429]. All together this experimental evidence support the notion of an intra-embryonic origin of mammalian hematopoiesis[384,430]. However, based on these results, it is not possible to completely rule out the potential contribution of the yolk sac to adult hematopoiesis by providing some sort of dHSC precursor not detected in the assays performed so far. The formation of the AGM in the embryo follows a defined set of events and it has been best described in non-mammalian vertebrates. In mice, hematopoietic clusters and endothelium of the dorsal aorta originate from lateral

mesoderm[476], which corresponds to the dorsolateral plate (DLP) in amphibian and the posterior lateral mesoderm (PLM) in zebrafish, two animal models extensively used to decipher early embryo development. The adult hematopoietic program in the AGM starts with the specification of DLP cells into endothelial (expressing Flk1 and Fli1) and hematopoietic (marked by SLC, GATA2 and LMO2 expression) cells prior the migration to the midline[477,478]. Subsequently, DLP cells migrate towards the midline and give rise to the dorsal aorta. At this stage VEGF signaling plays a key role in the modulation of SCL and GATA2 expression as well as the migration of Flk1+ endothelial cells to the midline[479,480]. Finally, arterial specification occurs coinciding with the emergence of adult hematopoietic cells. It has been demonstrated that morphogenesis of the aorta is linked to arterial and hematopoietic development [481–483]. In zebrafish, arterial and hematopoietic programs are both controlled by Hedgehog, VEGF and Notch, whereas BMP4 appeared to be essential for the hematopoietic development only[484]. In mice, BMP4 and Sonic-hedgehog (Shh) play a role in the expansion of dHSCs in the AGM[485,486]. An extensive amount of experimental evidence support the pivotal role of the AGM as a source of dHSCs in the developing embryo. AGM explants can autonomously generate bona fide CFU-S *in vivo*[427] and cells isolated from the AGM after E10 have been shown to successfully provide long-term reconstitution in adult mice[429]. Likewise, *in vitro* culture of AGM cells confirmed their ability to generate dHSC after E10.5[473], which expand up to 150-fold when exposed to recombinant growth factors[450,487]. Moreover, *in vitro* analysis of embryonic tissues before the establishment of circulation (E7-8.5) showed that only cells originating from the P-Sp/AGM region have the ability to generate multipotent progenitors both in mice[488] and in humans[489]. Conversely, only erythro-myeloid progenitors were detected from yolk sac-derived cells[488,489]. Furthermore, no LTR activity was reported for any of these tissues when transplanted into regular adult mice. However, only P-Sp/AGM cells

were capable of long-term reconstitution when engrafted into immunodeficient *Rag* $\gamma c^{-/-}$ mice, supporting the idea that immature dHSC precursors are localised in the AGM, even though a further maturation step, including the acquisition of MHCI expression, is needed to become fully functional[472].

Several lines of evidence support the idea that hematopoietic progenitors are generated in a short window of development. Fate mapping of labelled cells expressing VE-Cadherin[441] or RUNX1[395] showed labelling of 100% of adult HSCs only when cre recombinase was provided between E8.5 and E9.5. Moreover, the number of progenitors in AGM massively increases at E9 and E10, reaching a peak at E10.5 and decreasing thereafter. In the AGM, newly generated dHSCs are localised in intra-aortic hematopoietic clusters (IAHC) on the ventral floor of the dorsal aorta. IAHC have been observed in many species and they are characterised by the expression of both endothelial and hematopoietic markers[386,424,434,440,490]. In humans, the intra-embryonic hematopoietic wave is marked by the appearance of IAHC at CS13 on the ventral wall of dorsal aorta in the AGM region[491]. This formation lasts until CS17 and as opposite to mice development, it is restrained in the floor of dorsal aorta in the pre-umbilical area[424,425].

2.3.3 Alternative sites of embryonic hematopoiesis

2.3.3.1 Placenta

In mice and humans the placenta has been described as a hematopoietic niche, favourable to dHSC maintenance and expansion[436,487]. However, it is unclear whether the detected cells are newly generated dHSCs or circulating progenitors colonising the tissue.

In mice, dHSC are detected in the placenta simultaneously to dHSC emergence in the AGM[436,492] and hematopoietic progenitor can be detected as early as E8-9.

Nevertheless, fully functional dHSCs showing repopulating potential arise only at E10.5-11 and after a rapid expansion around E12.5 they reduce in numbers by E15.5, suggesting potential translocation to the fetal liver[436,492]. Furthermore, hematopoietic clusters have been identified in the placenta of *Ncx1*$^{-/-}$ mice, a model lacking functional circulation and therefore suggesting an independent generation of dHSCs in this site[493]. However, placenta explants cultures failed to give rise to definitive HSCs[494]. In the human placenta, hematopoietic cells are detected as early as week 4-5, and they include CD34hi CD45lo CD38- progenitors containing high CFU-Cs and CD34+ CD45lo progenitors committed to erythroid and myeloid lineages[495]. At this stage human placenta represent also a site for erythrocytes maturation[496]. Transplantation experiments using immunodeficient mice (NSG) have demonstrated that the first human definitive HSC can be detected in the placenta as early as week 9[487,497]. As opposite to mice development, the appearance of dHSC in human placenta occurs significantly later than AGM, implying that human placenta is not a site for HSCs generation. However, the exact location, contribution and significance of HSCs in this site are not known.

2.3.3.2 Umbilical cord

An additional site that has been evaluated as a source of dHSC generation is the umbilical cord. RUNX1-expressing cell clusters have been detected in the murine umbilical cord[498,499]. Also, very rare dHSCs can be identified in umbilical cord vessel by E10.5[423]. Nevertheless, explants culture of murine umbilical cord cells did not give rise to any definitive HSC.

2.3.3.3 Fetal liver

The fetal liver is considered the main site for dHSC maturation and expansion. Hematopoietic stem cells generated in the AGM migrate and colonise the fetal liver

between E11.5 and E12.5, coinciding with their appearance in the embryonic circulation. Here, dHSCs undergo a massive expansion mediated by angiopoietin-like factors and SOX17[500,501]. From E16.5, dHSCs start migrating from the fetal liver and colonise the bone marrow, where they will localise in the hematopoietic niche, which represents the only source of HSCs in the adult and supports their maintenance and expansion throughout life.

Very little is known about human fetal liver and most of the knowledge about its organization and function is based on experimental evidence obtained from the murine model. Human fetal liver emerged as a diverticulum of the embryonic gut at CS10. Starting from CS10 the liver contains primitive yolk sac-derived erythrocytes and CD45+ hematopoietic cells. Afterwards, by CS13, the human fetal liver contains CD34+ CD45+ cells[425], most likely originating from the second wave of yolk sac-derived EMP hematopoietsis[410,412], even if strong experimental evidence is missing to fully support this hypothesis. A fraction of these cells might also migrate from the IAHC in the AGM, which are known to emerge at the same stage of embryo development (CS13). However, the first human dHSC with transplantable potential appears in the fetal liver only at CS17[426].

2.3.3.4 Fetal spleen

In mice, the fetal spleen is an organ of mesoderm origin[502], which shows hematopoietic capacity exclusively during embryonic life, and subsequently become a secondary lymphoid organ in the adult. From E12 hematopoietic cells can be detected in the fetal spleen. This pool of hematopoietic cells consists in F4/80+ macrophages and large nucleated basophilic erythroblast[503]. The time of appearance and their independency from the transcription factor MYB support their primitive origin[37]. After circulation is established, the fetal spleen is colonised by circulating dHSCs[504,505], whose phenotype

and repopulation potential closely resemble their fetal liver counterparts[253]. However, HSCs in the fetal spleen are infrequent[504]: at E15.5 the HSC frequency of engraftment is 5 HSCs per spleen and this value increases between E16.6 and E18.5[253,505]. Finally, no hematopoietic niche has been described in the fetal spleen, supporting the maintenance, expansion and survival of HSCs.

2.4 Circulation in hematopoietic development

The contribution of circulation in the emergence of definitive HSCs and its involvement in the spatiotemporal organisation of the embryonic hematopoietic system has not been fully elucidated yet. In mice, yolk sac vasculature connects to the embryo body at E8 and a fully functional circulation starts to be established at E10. Originally, the role of circulation has been investigated mainly using two mouse models: the Ncx1-null embryos, which fail to initiate heart beating and die by E10[392], and the Rac1 mutant embryos, in which cell migration is impaired[475]. Ncx1-null embryos showed normal erythrocytes and CFC-C count in the yolk sac but no hematopoietic progenitors were detected in the embryo body[392]. Likewise, Rac1 mutant embryos had normal CFU-C in the yolk sac, but no IAHC formation or fetal liver hematopoiesis was detected[475].

These results led to the hypothesis that intra-embryonic hematopoiesis is strictly dependent on migration of yolk sac-derived progenitor that colonise the AGM and give rise to multipotent dHSCs. However, an alternative approach was later proposed, and the attention was focused on the contribution of physical and mechanical forces produced by the blood flow and their ability to influence and modulate endothelial cells function. Indeed, initial observations supported the idea that endothelial cells express mechanical receptors that induce transcriptional and phenotypic changes in response to heart beating and blood flow[506]. Furthermore, nitric oxide (NO) showed to be involved in VEGF-induced angiogenesis[507] and it was demonstrated that NO levels in endothelial

cells could be modulated by shear stress[508]. On these premises, a new hypothesis was proposed, based on the idea that biochemical forces resulting from blood flow may be responsible for the induction of the aortic endothelium to generate hematopoietic cells through NO signalling[509,510]. It was demonstrated that shear stress can induce RUNX1 and MYB expression and increase CFU-C production *in vitro*, both in AGM cells and embryonic stem cells (ESC), and this effect was abrogated by NO signalling inhibitors[509]. Moreover, *in vivo* administration of NO inhibitors abrogated intra-aortic clusters formation and reduced CFU-S, CFU-C and dHSC production in the AGM[509]. In addition, further *in vivo* evidence was provided from the "silent-heart" mutants in zebrafish, where the absence of heart beating induces a reduction of RUNX1+ and CD41+ hematopoietic progenitors. The re-establishment of a regular NO signalling by the administration of NO donors was sufficient to revert the phenotype[510,511].

In conclusion, even if mouse models lacking a functional circulation/migration suggested that the translocation of a yolk sac-derived hematopoietic ancestor might be needed to initiate AGM hematopoiesis, the modulation of NO signaling, both *in vitro* and *in vivo*, appeared to play an essential role in the establishment of a definitive hematopoietic program. More interestingly, evidence was provided demonstrating that a yolk sac-independent hematopoiesis in the AGM was achieved in the absence of circulation by re-establishing a functional NO signaling.

2.5 Endothelial origin of hematopoietic cells

It is commonly accepted that blood cells originate from embryonic endothelium and this has been demonstrated in many experimental models, including, zebrafish, frogs, chicks, mice and humans[411,444,446,512,513]. The hemogenic endothelium (HE), a hematopoietic precursor with endothelial features present in hematopoietic niches in the developing embryo, gives rise to newly generated hematopoietic progenitors through a

process called endothelial-to-hematopoietic transition (EHT)[445]. In mice, EHT is a multistep process characterised by different stages of maturation of the hematopoietic progenitors[388,450,461]. Mouse hematopoietic progenitors in the embryo or differentiated *in vitro* from embryonic stem cells (ESCs) initially express the endothelial marker VE-Cadherin, and subsequently acquire the expression of CD41 and CD45[399,445,514,515]. Even if expressing several endothelial markers, these cells do not display any endothelial potential anymore, and they are fully committed to the hematopoietic lineage. In mice AGM, EHT is a process driven by the co-operation of several transcriptional programs, including the transcription factors RUNX1 and SOX17, and it is antagonised by the expression of HOXA3[442,516,517].

In humans, KDR+ CD34- mesoderm derived precursors give rise to the endothelial cells forming the dorsal aorta upon acquisition of CD34+ expression[518]. From CS13, CD34 is expressed in both the aortic endothelium and the intra-aortic hematopoietic clusters (IAHC)[425]. These CD34+ CD45- cells isolated from AGM and umbilical vein give rise to both myeloid and lymphoid progeny when cultured *in vitro*[519]. Therefore, hematopoietic precursors (HE) are present in the CD34+ CD45- fraction of the aortic endothelium and they undergo EHT to give rise to fully committed hematopoietic cells. However, the CD34+ CD45- cells are an heterogeneous population that contain cells other than endothelial, as shown in *in vitro* differentiation experiments using human pluripotent stem cells (PSCs), where the expression of CD43 marks a subset of CD34+ CD45- cells already committed to the hematopoietic fate[520]. Therefore, CD34+ CD45- cells in human AGM may contain a fraction of precursors already committed to the hematopoietic lineage, and even if some of the mechanisms responsible for EHT have been described[393], further studies are needed to clarify the set of events leading to dHSCs and hematopoietic progenitors formation from the embryonic endothelium in humans.

In conclusion, even if the endothelial origin of the first definitive HSC in the human embryo represents the most commonly accepted hypothesis, further experimental evidences are missing to unequivocally rule out alternative scenarios[388,426]. For instance, the observed penetration of human IAHC into the endothelial lining of the dorsal aorta [425] might be explained by: i) excessive EHT in the aortic endothelium; ii) migration of cells undergoing EHT towards the umbilical vein, as observed in zebrafish[443,444]; iii) trans-endothelial migration of hematopoietic precursors sitting underneath the endothelium towards the lumen of the dorsal aorta (no EHT)[408,451] (Figure 1-6). Moreover, the expression of angiotensin converting enzyme (ACE), a feature of hematopoietic cells in the fetal liver, has been reported on few cells scattered beneath the endothelium of the human dorsal aorta in CD34- cells. These cells might represent precursors of IAHC that acquire CD34+ expression when integrated in the endothelium (potential non-endothelial origin)[521].

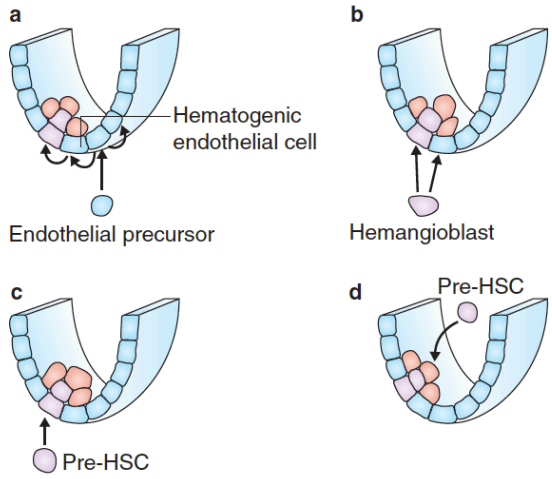


Figure 1-6. Potential mechanisms of dHSC origin in the intra-aortic hematopoietic clusters (from Medvinsky et al, 2011). (a) An endothelial precursors give rise to hemogenic endothelium (HE) that undergo EHT and differentiate into pre-HSC and dHSC. (b) The hemangioblast gives rise to hematopoietic precursors and endothelial cells independently. (c) Non-endothelial pre-HSC lying underneath the endothelial lining matures into dHSC and enters the circulation. (d) A pre-HSC of non-endothelial origin migrates through circulation and integrates into the endothelial lining.

2.5.1 The hemangioblast

In 1932, Murray first described the existence of a mass of cells of mesoderm origin containing both endothelial and hematopoietic cells and named it hemangioblast[522]. This concept arose as opposed to the Angioblast described earlier by Sabin, a similar mass of mesoderm cells constituted exclusively of endothelial cells[348]. This idea was consistent with the hypothesis that blood islands in the yolk sac might consist in clonal structures and that a bi-potent progenitor with both endothelial and hematopoietic potential should exist[348]. More recently, even if the clonal origin of the yolk sac blood islands has been revised, the existence of the hemangioblast as a clonal progenitor for endothelial and hematopoietic cells gained more and more interest. Early attempts to demonstrate the existence of such progenitor in mice and avian *in vivo*, were unsuccessful[523,524]. However, in the late 90s a clonal multi-potent precursor was identified as a blast colony-forming cell (BL-CFC) in *in vitro* differentiation assays using mouse embryonic stem cells (ESCs)[397,525]. The phenotype of this BL-CFC was established as KDR+ Bry+ VE-cad- CD31- KIT- CD45- CD34-, and these cells displayed primitive hematopoietic cells potential, introducing the idea that the hemangioblast might represent the progenitors of primitive hematopoiesis[526]. Subsequently, BL-CFCs were identified in murine posterior primitive streak at E7[398], in zebrafish[527] and in human ESC-derived mesoderm[528]. Nevertheless, conclusive experimental evidence that the hemangioblast can give rise to both endothelial and hematopoietic cells *in vivo* in higher vertebrates is still missing. Of note, lineage tracing experiments based on Flk1-cre or Tie2-cre mouse models failed to demonstrate the existence of the hemangioblast *in vivo*[469,529], even though the strong limitations of the chosen mouse models need to be taken into account[389].

2.5.2 The hemogenic endothelium

The term hemogenic endothelium (HE) refers to cells with endothelial phenotype, gene signature and morphology, which *in vivo* localise to the endothelial layer of blood vessels and have the unique ability to give rise to HSPCs. It has been formally proven, both *in vitr*o and *in vivo*, that blood cells originate from HE[399,440,441,444–447,530] via endothelial-to-hematopoietic transition (EHT)[445]. HE cells express the endothelial markers Ve-Cadherin[458,513] and CD31[458] as well as transcription factors involved in hematopoietic differentiation such as RUNX1[513] and GATA2[531,532]. Moreover, HE cells localise in specific sites in the developing arterial vasculature including the ventral wall of the dorsal aorta in the AGM and other hematopoietic niches (umbilical cord veins, brain vasculature, e.g.). All these aspects suggest that the HE might originate from endothelial cells, even though this has not been formally proven yet. In fact, the original model describing the HE was based on the idea that HE cells are bi-potent progenitors with both endothelial and hematopoietic potential (Figure 1-7). However, this concept has been recently challenged by showing that HE cells are already committed to the hematopoietic fate and no endothelial potential was observed both *in viv*o[533] and *in vitro* using mouse and human PSCs cultures[533–535]. The lack of endothelial potential in clonogenic cultures of mouse and human HE cells further supports this hypothesis[534,536].

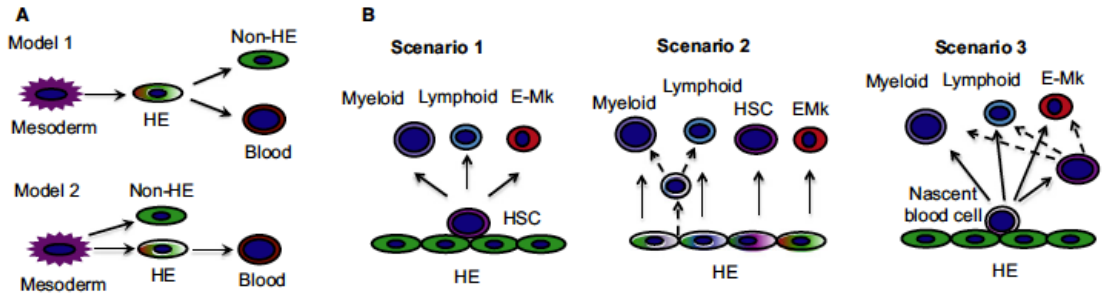


Figure 1-7. Potential models of blood cells specification from hemogenic endothelium (from I. Slukvin, 2016[537]). (a) Alternative models proposing the hemangioblastic potential of the HE that gives rise to both endothelial and hematopoietic cells (top) or the HE as a transitional stage of progenitors already committed to the hematopoietic fate (bottom). (b) Potential scenarios of blood cells specification from HE. HE differentiates into HSCs that give rise to all other lineages (1). HE is heterogeneous and specification of blood cells occurs at the HE stage (2). HE can generate all hematopoietic lineages including HSC (3).

Further insights on the development and heterogeneity of the human hemogenic endothelium have been provided by the *in vitro* differentiation of human PSCs. During *in vitro* differentiation of human PSCs, hematopoietic cells follow the same path observed *in vivo* during embryonic development and EHT happens in Flk1+ mesodermal cells, from which hematopoietic cells have been observed to originate[399,445]. In human PSC *in vitro* cultures, HE and arterial endothelium are already specified at day 8 of differentiation and HE and non-HE can be reliably discriminated by the expression of specific surface markers: CD43 marks the appearance of hematopoietic committed cells[520,526], whereas CD73, DLL4 and CXCR4 are exclusively expressed in non-HE cells only[534,538,539]. Furthermore, in human PSC differentiation cultures a precursor with endothelial but not hematopoietic potential can be detected[536] and a subset of mesoderm lacking blood-forming potential that gives rise exclusively to endothelial cells can be identified based on the expression of KDR[534]. Therefore, the exact origin of hemogenic endothelial cells has not been completely elucidated. Further observations in mice have shown that endothelial progenitors can migrate from the yolk sac to intra-embryonic sites in the dorsal aorta at E7-7.5[540,541] and the blood specification of these cells is impaired by

RUNX1 silencing[540]. Moreover, RUNX1 expressing cells labelled at E7.5 showed to contribute to the dorsal aorta formation and HSC generation[395,541]. All together these evidences might suggest an extra-embryonic origin of HE[389].

HE is heterogeneous and it can vary among different hematopoietic niches as well as within the same site. For instance, only the ventral domain of the dorsal aorta generates definitive HSCs, whereas the dorsal domain gives rise only to multipotent progenitors, but no dHSCs[448]. Further evidences of HE heterogeneity have been provided by *in vitro* differentiation of human PSCs. In this experimental setting, the fate of different HE cells appeared to be already defined in the early mesoderm. APLNR+ PDGFRα+ mesoderm expressing primitive streak genes gives rise to the hemangioblast, which subsequently differentiate to primitive erythrocytes (expressing embryonic globin) through an intermediate HE step via EHT[534,536]. Conversely, HE originating from mesoderm expressing TAL1, ETV2 and GATA2 but lacking expression of primitive streak genes, has shown a broad myelo-lymphoid differentiation potential[467,534,542]. In addition, continuous imaging of human PSCs cultures showed that at least two waves of EHT occur during *in vitro* differentiation[543] and the overexpression of specific transcription factors in human PSCs can drive the differentiation of HE with different potential[544] (Figure 1-7). It has been clearly demonstrated that the hemogenic endothelium is not specific for any embryonic hematopoietic program, and experimental evidences have been provided to prove the hemogenic endothelial origin of mouse E8.25 yolk sac EMPs[407], E9.5 yolk sac LMPPs[413,414] and E10.5 AGM-derived definitive HSCs[447]. However, the presence of hemogenic endothelium in the yolk sac and its involvement in the primitive hematopoietic program is not fully elucidated yet. HE has been identified in the yolk sac[529], but primitive hematopoietic cells arise in the yolk sac as early as E7.5 from masses of cells expressing endothelial markers[399,545]. Moreover, at E7.5 vascularisation has not occurred yet, and those hematopoietic precursors

expressing endothelial markers cannot be considered *bona fide* hemogenic endothelium. For this reason the term hemogenic angioblast has been proposed for these yolk sac-derived cells with primitive blood cells potential only[389].

In conclusion, the co-existence of hemangioblast and hemogenic endothelium in a unified model has been proposed[399], by which the hemangioblast gives rise to hemogenic endothelium in a SCL-dependent fashion and subsequently the HE differentiates into definitive hematopoietic cells in a process dependent on the transcription factor RUNX1.

2.6 Signalling pathways involved in embryonic hematopoiesis

Embryonic development of endothelium and hematopoietic cells are closely related and shared common signalling pathways. Experimental evidences support the idea that the fate of nascent HSCs is not strictly pre-determined at the HE state, but is consistently dependent on external signals provided by the surrounding microenvironment. Newly generated blood cells budding from the endothelial layer in the AGM is a step-wise process controlled by factors provided by the surrounding mesenchyme, endothelial cells and IAHC[444]. Moreover, the disruption of sub aortic mesenchyme blocks initiation of RUNX1 expression and the formation of IAHC without affecting vessels formation and arterial identity in the avian model[546].

Based on *in vitro* and *in vivo* studies, several signalling pathways have been shown to play a role in the activation of specific transcriptional programs driving hematopoietic cells specification.

2.6.1 The KDR/VEGF axes

KDR (also known as VGFR2/Flk1) is a class IV receptor tyrosine kinase (RTK), which binds with high affinity to vascular endothelial growth factors (VEGF) and initiates

various intracellular signalling pathways[547]. KDR is mainly expressed in blood vascular endothelial cells and plays a crucial role in the formation and maintenance of the vasculature[547].

During embryogenesis, KDR plays a fundamental role in the formation of dorsal aorta in various species[548]. In humans, KDR+ CD34- cells were detected at CS10[518], a phenotype resembling the hemangioblast described in other contexts[528,537,548]. Moreover, endothelial cells and IAHCs in the dorsal aorta at CS14-15 express KDR and produce its ligand VEGF[466]. KDR is also expressed in early mesoderm in human PSCs cultures *in vitro*. KDR expression can be used to discriminate between mesoderm with hematopoietic potential (KDR^{bright}) and mesoderm giving rise exclusively to endothelial cells (KDR^{dim})[534]. Within the KDR+ mesoderm two subsets can be identified based on the expression of CD235a (glycophorin A) as early as day 3 of differentiation. Further analysis KDR+ mesoderm revealed that KDR+ CD235a+ fraction is enriched in cells with primitive hematopoietic potential, whereas T lymphocytes potential was detected in the KDR+ CD235a- population[535].

2.6.2 The KIT/SCF axes

KIT is a member of RTK-III (class III receptor tyrosine kinases). KIT is express in hematopoietic stem and progenitors cells (HSPCs) in the bone marrow, as well as in melanocytes and mast cells. The interaction of KIT with its cognate ligand stem cell factor (SCF) is crucially involved in hematopoiesis, melanogenesis, spermatogenesis and mast cell development[547].

SCF is expressed by fibroblast, stromal cells and endothelial cells and exists both in soluble and membrane bond form. Differential effects of the two isoforms have been reported from *in vitro* and *in vivo* studies[549].

The emergence of dHSC in the early embryo requires SCF signalling[259,461], and the interaction with its cognate receptor KIT plays an important role in HSC maturation in the mouse AGM[461,550]. Indeed, addition of recombinant SCF to explants culture of mouse dorsal aorta increases HSC generation from the ventral compartment and induces HSC formation in the dorsal one[550]. In human embryo, KIT is expressed in the IAHC and in newly generated HSCs[449,551], suggesting the same important role for SCF/KIT signaling in human embryonic hematopoiesis.

2.6.3 The FLT3/FLT3L axes

The receptor tyrosine kinase Flt3 (also known as Flk2) is expressed in HSPCs in the bone marrow both in mice and humans. However, mouse long-term reconstituting stem cells (LTR-HSC) do not express Flt3 and its up-regulation is accompanied by loss of self-renewal capacity[182,552]. Conversely, a fraction of LTR-HSCs in human bone marrow and cord blood express Flt3 and most of the CD34+ HSCs capable of reconstitute immunodeficient mice (NOD/SCID) are Flt3+[183,184]. The interaction of Flt3 with its cognate receptor Flt3L has a fundamental role in HSPC and B cell progenitors expansion in the bone marrow, and it represents an essential regulator of DC development and homeostasis[17,34,45,185,186]. During embryogenesis, FLT3 expression marks mouse embryonic HSC precursors[553] and both FLT3 and its ligand FLT3L are expressed in the IAHCs and surrounding endothelium in human AGM[466]. However, there is no evidence of a specific role of FLT3/FLT3L in the development of the hematopoietic system in the early phases of embryonic life.

2.6.4 BMP4

Bone morphogenic protein 4 (BMP4) is a member of the transforming growth factor beta (TGFβ) superfamily. BMP4 is involved in bone and cartilages formation as well as in the early stages of human embryonic development.

During embryonic hematopoiesis, the emergence of definitive HSCs strictly requires BMP4, which has been shown to contribute to the formation of the AGM niche in the early embryo[484,550].

Moreover, BMP4 promotes hematopoietic differentiation from human PSCs *in vitro*[554,555] and induces the expression of tenacin C and fibronectin[556], which further contribute to the establishment of the hemato-endothelial program in human PSCs cultures[467,557]. *In vivo*, BMP4 is highly expressed in the ventral mesenchyme underneath the dorsal aorta and it is regulated by FGF[485,558]. Indeed, the inhibition of FGF signaling in zebrafish enhances BMP4 expression triggering the definitive hematopoietic program[558]. However, BMP4/TGFβ signaling appeared to be crucial for hematopoietic induction in a very short window of time. In fact, it has been shown that BMP4 negatively regulates HSC maturation[550] and its inhibition occurs in the IAHC after endothelial-to-hematopoietic transition[550,559]. Inactivation of BMP4 signalling during hematopoietic development is achieved by expression of the antagonist Noggin, which is potentiated by a positive feedback loop by the expression of Sonic-Hedgehog (Shh)[550].

2.6.5 Notch signalling pathway

During embryogenesis the dependency on Notch signalling represents a very reliable approach to discriminate between the different hematopoietic programs. Many experimental evidences clearly established that Notch signalling plays a fundamental role in the induction of the definitive hematopoietic program, and this notion has been

confirmed in various models, such as zebrafish, chicks and mice[406,446,560]. Conversely, Notch appeared to be dispensable for the initiation and progression of the yolk sac as well as EMP-dependent hematopoiesis. Its role in the LMPP program has not been addressed yet. *Notch*$^{-/-}$ mouse embryos develop normal CFC-C in the yolk sac but very limited in the embryo body[560,561]. Furthermore, *wt:Notch*$^{-/-}$ chimera did not show any hematopoietic cells with *Notch*$^{-/-}$ genotype in the adult mouse[560]. Notch and its ligands Jag1 and Jag2 are preferentially expressed in the ventral floor and in intra-aortic clusters on the dorsal aorta of E10.5 embryos[562,563]. It has also been demonstrated how Notch control GATA2 expression[562,563], which control RUNX1 expression[564]. Indeed, overexpression of GATA2 or RUNX1 could partially restore AGM hematopoiesis in Notch and Jag1 knockout mice[562,563]. In addition, Notch signalling plays a role in the specification of arterial and venous endothelium[565]. In chicks, uncontrolled activation of Notch leads to "arterialization" of the venous endothelium[406]. Murine PSC-derived hematopoietic progenitors lack the expression of Notch when compared to embryonic hematopoietic niches, an observation that might explain the limited potential of these *in vitro* generated cells [566]. However, human PSCs culture showed that even if important for blood formation, Notch signalling is not sufficient to successfully induced the generation of self-renewing definitive HSCs[539,567]. The precise mechanism by which Notch promotes definitive hematopoiesis is not fully elucidated, but recent publications may suggest a potential role in the development of the hemogenic endothelium in the AGM[195,568].

2.6.6 Activin/Nodal and Wnt/β-catenin pathways

Nodal/Activin and Wnt/β-catenin are two very important signalling pathways involved in the development of the early embryo. The establishment of active Nodal and Wnt signalling gradients represents a key mechanism by which the tissues of the developing

embryo are specified and differentiate. Based on these observations, the effect of the modulation of Nodal/Activin and Wnt/β-catenin signalling on embryonic hematopoiesis has been tested in human PSCs differentiation experiments. This *in vitro* approach demonstrated how Nodal/Activin signalling pathway promotes primitive hematopoiesis and inhibits the establishment of a definitive program[535,569]. Conversely, Wnt/β-catenin pathway appeared to promote definitive hematopoiesis while abrogating the primitive hematopoietic program[535,569]. Very interestingly, the modulation of both pathways was effective only in a very narrow temporal window of 72 hours of differentiation, suggesting a direct effect on mesoderm formation and patterning. These observations were consistent with previously reported evidence of a fundamental role of Wnt in the emergence of HSCs *in vivo*[570,571]. More importantly, these results are compatible with the spatial organisation of the developing embryo, where the mesoderm cells committed to the primitive hematopoietic fate are exposed to an active Nodal signalling as well as a Wnt inhibition[572].

2.6.7 Inflammatory cues

A further example of extrinsic factors involved in hematopoietic development during embryogenesis is the contribution of pro-inflammatory mediators to the generation of definitive HSCs in the AGM. Several examples have been reported in different models. Interferon produced by embryonic myeloid cells and retinoic acid signalling play an important role in the formation and specification of hematopoietic progenitors in mouse yolk sac and AGM[573–575]. Moreover, TNF-mediated activation of NF-kB and Notch signalling pathways has been reported to be involved in HSC development both in zebrafish and mice[576,577]. Finally, the use of retinoic acid in human PSC cultures *in vitro* induces the differentiation of definitive HE characterised by the expression of HOXA

cluster genes, a key feature of dHSC maturation and expansion[578]. However, no positive effect on self-renewal and repopulation capability has been reported.

2.7 Transcription factors involved in embryonic hematopoiesis

2.7.1 RUNX1

The transcription factor RUNX1 is a master regulator of dHSC development in many species[481,579,580]. RUNX1 is preferentially expressed in hematopoietic cluster in the dorsal aorta[499] where it plays a fundamental role in the regulation of EHT[442]. Indeed, RUNX1 activates its downstream target GFI1, which contributes to the suppression of the endothelial program and promotes hematopoietic differentiation[581]. RUNX1 promotes EHT in combination with the transcription factor GATA2[582], and it is negatively regulated by the expression of HOXA3[516]. *Runx1*$^{-/-}$ mice display a normal yolk sac erythropoiesis but no dHSC generation and embryos die around E12.5[583,584]. IAHCs are absent in *Runx1*$^{-/-}$ mice[499,585], and an accumulation of mesanchymal cells has been observed beneath the dorsal aorta, suggesting a potential impairment of EHT[499]. Impaired EHT has been reported in *Runx1* knock out zebrafish[444], and a dysfunctional hemogenic endothelium with no hematopoietic potential has been described in *in vitro* differentiation of *Runx1*$^{-/-}$ mouse embryonic stem cells (ESCs)[399]. Moreover, the overexpression of RUNX1 is sufficient to re-establish functional definitive hematopoiesis in *ex vivo* cultures[586]. Dose and time of RUNX 1 expression is also a key aspect, as demonstrated by the decreased hematopoietic differentiation in the AGM and the early appearance of dHSC in the yolk sac in RUNX 1 or GATA2 haploinsufficient mice[531,587]. Furthermore, the induction of RUNX1 expression has been reported in response to biomechanical forces both in AGM cells and in ESC-derived hematopoietic cells, resulting in an increased production of hematopoietic progenitors *in vitro*[509].

2.7.2 MYB

Another transcription factor intimately involved in the establishment of the definitive hematopoietic program during embryogenesis is MYB. The dependency on MYB represents a reliable approach to discriminate between the primitive versus the definitive origin of differentiated hematopoietic cells. Indeed, MYB expression has been reported in definitive progenitors and in EMPs[419], but not in cells originating from the primitive hematopoietic program[385]. Primitive hematopoiesis is not affected in *Myb*$^{-/-}$ mice[588–590], and primitive erythroblast, megakaryocytes[591] and EMP-derived macrophages[37] develop normally in these animals. However, the absence of MYB completely abrogates the establishment of the definitive hematopoiesis and the embryos die at E16[588,590]. Moreover, MYB expression has been identified as a key event following EHT during dHSC differentiation from arterial endothelium in zebrafish[446]. Of note, hematopoietic differentiation of human PSCs using a MYB-reporter line has shown that *in vitro* differentiated cells do not express high levels of MYB after EHT, which might limit the differentiation of proper dHSCs[592].

2.7.3 SOX17

SOX17 is a member of the SOX (SRY-related HMG-box) family of transcription factors, mainly involved in regulating pluripotency, self-renewal and differentiation processes in embryonic development. SOX17 has been shown to play a critical role in the emergence of HSCs during embryonic development and to promote arterial and hemogenic endothelium specification[516]. Once EHT occurred, SOX17 expression is abrogated in order to allow the initiation of the hematopoietic program[593]. In mouse and human PSC-derived hemogenic endothelium, SOX17 has been used to identify the emergence of the definitive hematopoietic program[569,594]. In human PSCs *in vitro* cultures, overexpression of SOX17 in CD34+ CD43- cells appeared to promote the

differentiation of Ve-Cadherin+ CD43+ CD45- hematopoietic progenitors with blood cells potential upon down-regulation of SOX17 expression[595].

2.7.4 HOXA genes

HOX genes are a group of very conserved genes that code for transcription factors involved in the regulation of the early embryo development. *HOX* genes are part of the homeobox transcription factors genes, and in human they are classified in four different clusters (HOXA, HOXB, HOXC and HOXD).

HOXA genes are already expressed at the primitive streak stage in the embryo[596] and they mark intra-embryonic hematopoiesis during mesoderm patterning. Hematopoietic progenitors isolated from umbilical cord or fetal liver express high levels of *HOXA* genes, whereas *in vitro* generated PSC-derived hematopoietic progenitors failed to do so[578,597], suggesting an inefficient activation of the definitive hematopoietic program. However, it has been recently reported that *HOXA* genes expression can be induced in human PSC-derived hematopoietic progenitors by modulating the Activin/Nodal as well as Wnt/β-catenin signalling pathways, more closely recapitulating the definitive hematopoietic program taking place in the early embryo[597]. Furthermore, lineage conversion studies, an approach by which the re-programming of pluripotent as well as terminally differentiated cells is achieved by forced expression of specific transcription factors, have significantly contributed to highlight the importance of *HOX* genes in the development of definitive hematopoiesis. Activation of *HOXA* genes appeared to have a strong impact on the expansion of mouse bone marrow HSCs[598,599] and in the temporal regionalisation of embryonic mesoderm[600]. Moreover, the overexpression of HOXB4 in mouse ESCs in association with Notch signalling activation conferred multilineage long-term repopulation abilities to *in vitro* differentiated hematopoietic progenitors[601]. Nevertheless, forced expression of HOXA5, HOXA7 and HOXA9 in human PSCs-

derived progenitors was insufficient to convert them into fully functional definitive HSCs[578].

2.7.5 HOXA3

HOXA3 is one of the members of the *HOXA* genes, which plays a pivotal role in the cell fate decision between the endothelial and the hematopoietic lineages. Overexpression of HOXA3 has been shown to inhibit the pro-hematopoietic transcription factor RUNX1 and promote the endothelial program[516]. Conversely, the down-modulation of HOXA3 allowed the RUNX1-mediated activation of the hematopoietic program at the expense of the endothelial pathway[516].

2.8 The study of embryonic hematopoiesis: a technical perspective

The understanding of the key events driving hematopoietic development in the human embryo as well as the spatiotemporal organisation of the embryonic hematopoietic system is closely related to the availability of reliable *in vitro* and *in vivo* assays. Advances in technology enabled a more accurate interpretation of the early events driving embryonic hematopoiesis and the appearance of the first definitive HSC. For instance, the use of human induced pluripotent stem cells (iPSCs) provided a potential inexhaustible source of human embryonic tissues overcoming bioethical concerns. Moreover, the generation of immunodeficient mouse models capable of supporting human hematopoietic cells maintenance and differentiation (e.g. NSG, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ), allowed the *in vivo* assessment of the function and repopulation potential of *ex vivo* isolated or *in vitro* generated hematopoietic progenitors. Finally, the development of very powerful gene targeting techniques, such as CRISPR/Cas9, enabled the very rapid and efficient generation of human knock-out cells allowing the investigation of key factors involved in early hematopoiesis in

genetically tractable environment. The following section will provide an overview of the different technical approaches, both *in vivo* and *in vitro*, that contributed to a better understating of the human embryonic hematopoiesis.

2.8.1 Studying human embryonic hematopoiesis: *in vitro* approaches

Using an *in vitro* culture system in semi-solid matrices (Methylcellulose), colony-forming-units culture (CFU-C) were identified in the human embryo as CD34+ hematopoietic progenitors[425]. However, technical limitations prevented an accurate evaluation and identification of the embryonic tissues giving rise to these cells[425]. To overcome this issue, mouse embryonic hematopoiesis was studied using an *ex vivo* expansion of tissues explants before assessing hematopoietic potential[386,488]. The same approach was then transferred to the study of human embryos. Tissues isolated at CS10, before the establishment of a functional circulation, demonstrated that while the yolk sac has the potential to generate myeloid and NK cells only, a broader spectrum of hematopoietic cells (including B and T lymphocytes) originate from the P-Sp/AGM region of the human embryo[489]. However, even if consistent with other models, the lympho-myeloid precursor identified *in vitro* in the p-Sp/AGM may not correspond to definitive HSCs. Therefore, to test the long-term repopulation ability of these cells and identify the true, rare HSC in the human embryo, *in vivo* studies needed to be performed, and the generation of a mouse model highly permissive for human cells (NSG) enabled this experimental approach.

2.8.2 Studying human embryonic hematopoiesis: *in vivo* approaches

The *in vivo* repopulation assay represents the gold standard technique to evaluate self-renewal and differentiation potential of hematopoietic progenitors in order to identify fully functional definitive HSCs. Using immunodeficient NSG mice, a meticulous

evaluation of the hematopoietic potential in the different tissues of the human embryo has been performed[426]. As a result, the first transplantable definitive HSC was identified in the AGM at CS14. Conversely, the yolk sac did not show any repopulation activity except for very rare events detected as early as CS16 (at least five days later than the AGM). Definitive HSCs were detected in the fetal liver after 7-8 weeks of gestation. These cells were still expressing the endothelial marker Ve-Cadherin, supporting the notion of an endothelial origin of the hematopoietic system[602]. After liver colonisation, which occurs between CS14 and CS17, HSCs can be detected in the placenta from 9 weeks of gestation[487] and in the umbilical cord, suggesting that they represent secondary sites for HSC development and they are colonised only after maturation and expansion in the liver. As compared to the mouse model where dHSCs appear almost at the same time in many tissues, the clear temporal separation of subsequent events leading to human dHSC generation represents a great advantage of performing *in vivo* studies with human embryonic tissues. Moreover, the differences in the time of HSC appearance observed comparing the *in vivo*[426] and *in vitro*[425,487,495] approaches, highlight the importance of *in vivo* experiments for the study of human embryonic hematopoiesis.

2.8.3 Modelling embryonic hematopoiesis using human PSCs

A substantial contribution to the understating of human embryonic hematopoiesis was made by the use of pluripotent stem cells (PSCs), in order to reproduce the early events of human embryogenesis. With the discovery of iPSCs by Prof. Yamanaka in 2006[603] and the successful re-programming of most of the human adult cell types to a pluripotent state amenable to *in vitro* differentiation[604], the establishment of reliable protocols to generate hematopoietic cells *in vitro* gained increasingly amounts of interest. In fact, besides acquiring a better understanding of the processes driving human embryonic hematopoiesis, this approach has the potential of generating patient-specific

HSPCs, and virtually all the cells types of the human immune system, for clinical purposes. *In vitro* differentiation of hematopoietic cells from mouse PSCs has shown a remarkable consistency with the mechanisms described *in vivo* in the embryo. Therefore, it is reasonable to consider human PSCs as a reliable approach to study hematopoiesis in the early stages of human embryonic life[605]. However, not all the aspects of *in vitro* differentiation of hematopoietic cells have been fully elucidated yet, and some conflicting evidence has been reported. For instance, while many publications support the idea that *in vitro* generated hematopoietic cells arise with different timing, mimicking the wave-like kinetic described *in vivo*[606–608], others reported the emergence of all blood cells types in the same time frame[609].

2.8.3.1 PSC-derived HSPC in vitro: differentiation potential and limitations

Two main strategies have been adopted to differentiate human PSCs into hematopoietic cells:

- co-culture of human PSCs with mouse bone marrow-derived stromal cell lines, among which OP9 demonstrate to be the most efficient option[610]
- induction of hematopoietic differentiation through embryoid bodies (EBs) formation by providing specific factors in a more controlled experimental setting[528,554,569]

From both approaches, a very extensive spectrum of hematopoietic lineages have been differentiated *in vitro* from human PSCs: erythroblast, macrophages, DC-like and monocyte-derived DCs, NK cells, neutrophils, eosinophils, basophils, megakaryocytes, B and T cells[610,611]. However, the main drawback of both approaches resides in the absence of reliable markers to firmly establish the primitive or definitive origin of these cells. Indeed, primitive and definitive hematopoiesis *in vivo* can be easily distinguished by their appearance in very specific locations and time windows, something that is not

reproducible *in vitro*. Due to the lack of specific markers identifying the primitive or definitive origin of the cells, the potential of the *in vitro* generated HSPCs is a commonly used approached. However, the fact that different hematopoietic programs can give rise to the same lineages adds a further layer of complexity to the reliable evaluation of the differentiated progenitors. Based on the evidence that most of the hematopoietic lineages, including T cells[414], can originate in an HSC-independent process, the most reliable indication of a successful induction of the definitive program is the appearance of Type2 adaptive B cells, a cell type exclusively derived from HSC.

To date, most of the *in vitro*-based protocols to generate HSPCs from PSCs appeared to mainly resemble primitive yolk sac hematopoiesis, providing a possible explanation for the absence of self-renewal and long-term repopulation observed in these PSC-derived progenitors. Indeed, *in vitro* generated cells have never shown repopulation ability comparable to dHSC isolated from AGM[426]. This comparison might suggest that not only the differentiation process still needs to be optimised and fully understood, but also other aspects, such as the maturation of pre-HSC into fully functional stem cells, have to be taken into account.

2.8.3.2 Embryonic hematopoietic programs in PSC-based in vitro cultures

So far, numerous approaches have been developed to generate HSPCs *in vitro* from human PSCs, which appear to be mimicking different stages of the embryonic hematopoiesis (Figure 1-8).

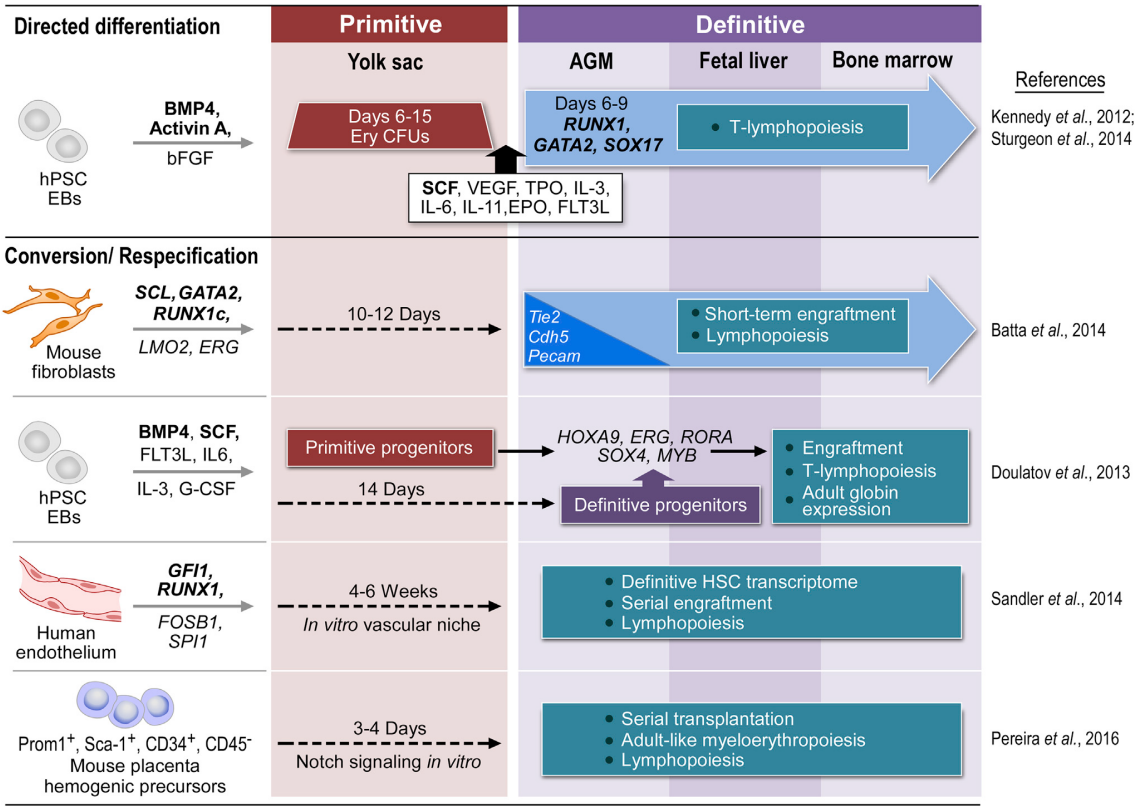


Figure 1-8. *In vitro* approaches to generate human hematopoietic cells (from Rowe et al., 2016[612]). The diagram summarise some examples of different approaches used to generate hematopoietic cells *in vitro*. Hematopoietic development was induced either by exposing pluripotent cells (PSCs) to morphogens/growth factors (Kennedy et al., 2012; Sturgeon et al., 2014) or by over-expressing defined sets of hematopoietic transcription factors in human PSCs (Doulatov et al., 2013) or terminally differentiated fibroblast (Batta et al., 2014), endothelial cells (Sandler et al., 2014) and mouse hematopoietic precursors (Pereira et al., 2016). The different methods were capable of recapitulating several stages of embryonic hematopoiesis, including the onset of primitive and definitive hematopoietic progenitors with variable engraftment and differentiation potential.

2.8.3.2.1 Mesoderm specification

In mammalian embryo mesoderm specification and hematopoietic differentiation are driven by the activation of several signalling pathways and specific factors are involved in this process, such as BMP4, FGF, WNT, TGFβ, Hedgehog, retinoic acid and Notch signalling[482,483,548,574,593]. *In vitro*, mesoderm patterning has been shown to recapitulate quite faithfully the *in vivo* process, and it depends on the presence of specific factors such as BMP4, FGF, Activin and canonical Wnt pathway[570,613–618].

Mesoderm committed to the hematopoietic fate is defined by the expression of specific genes (brachyury (T), MIXL1 and FOXF1, i.e.) and surface markers (KDR and

PDFGRα, i.e.)[418,537,619] and its specification to the different hematopoietic programs (primitive versus definitive) occurs in these early phases of mesoderm patterning. Indeed, mesoderm differentiated providing Activin and BMP4 signals appeared to resemble primitive hematopoiesis by the efficient generation of hematopoietic progenitors without repopulation potential, as described for yolk sac derived cells *in vivo*[570]. Moreover, the exposure of BMP4-driven *in vitro*-generated mesoderm to Activin inhibitors[569], Wnt agonists[535,620] and the combination of the two[597] resulted in the inhibition of the erythroyd-biased primitive program and the induction of more "definitive-like" progenitors, characterised by their potential to generate T lymphocytes. The commitment to different programs was already detectable at the mesoderm level based on the expression of KDR and CD235a[535].

2.8.3.2.2 Yolk Sac/Primitive hematopoiesis

PSC-derived BMP4-dependent mesoderm can be further differentiated into blast colonies (BL-CFC) by the presence of VEGF and bFGF in the culture medium. BL-CFCs represented the first identification of the hemangioblast *in vitro*[397,525], marking the onset of hematopoiesis[528,536,619]. *In vitro* PSC-derived hemangioblast is characterized by the expression of KDR, PDGFRα, apelin receptor[534,536,621] and CD235a[535], and has the potential to differentiate into the hematopoietic, endothelial and smooth muscle lineages[621]. The hematopoietic potential of these cells is limited to primitive erythrocytes, megakaryocytes and macrophages, and the terminal differentiation occurs through an hemogenic endothelium intermediate, as described previously in mice[399]. All together, these observations are consistent with the induction of a primitive hematopoietic program, resembling yolk sac-derived embryonic hematopoiesis. The same result can be achieved in alternative culture settings by exposing human PSCs, both as monolayer[467] or organized in embryoid bodies (EB)[555,570], to recombinant

cytokines cocktails, or by co-cultures of PSCs with OP9 mouse stromal cells[520]. When co-culture with OP9, a subset of PSC-derived CD34+ cells acquires expression of CD43, which has been identified as a reliable marker to discriminate by hematopoietic and endothelial cells in this experimental settings[520]. CD34+ CD43+ hematopoietic cells can be detected in all the described differentiation approaches and they showed to be capable of giving rise to a wide spectrum of erythroid and myeloid cells[534,569,597]. This would suggest that CD34+ CD43+ hematopoietic progenitors differentiated *in vitro* have a broader potential than *in vivo* primitive yolk sac-derived cells (limited to erythroblast, magakaryocytes and macrophages), but the absence of lymphoid progeny would exclude their definitive origin. These cells might correspond to yolk sac-derived EMPs described in mouse[410], and less extensively in humans[412]. However, the primitive or definitive origin of these cells has not been fully elucidated yet, even though the absence of expression of *HOXA* genes[578,597], a marker of AGM-derived definitive progenitors *in vivo*, would support the first option. Of note, even if EMPs are usually associated with the yolk sac, cells with the similar restricted potential can be detected within the Ve-Cadherin+ CD45+ population of the human AGM[449]. Therefore, these cells might be distinguishable from the yolk sac-derived EMPs only by the expression of *HOXA* genes.

2.8.3.2.3 AGM/definitive hematopoiesis and dHSC

The first definitive HSCs *in vivo* originates from the hemogenic endothelium localised in the dorsal aorta. Numerous publications reported the generation of PSC-derived hemogenic endothelium *in vitro*, whose phenotype has been identified as CD34+ CD31+ Ve-Cadherin+ CD73- CXCR4- DLL4- CD43-[534,539]. *In vitro*-generated hemogenic endothelium express RUNX1 and GFI1 in both mouse[499,581] and human cells[534,597]. Moreover, it has recently been reported the differentiation of human PSC-

derived hemogenic endothelium expressing *HOXA* genes, as described *in vivo* in the AGM[597]. HOXA+ hemogenic endothelium could also be subdivided in SOX17^{hi} cells, transcriptionally similar to the dorsal aorta endothelium, and SOX17^{dull} cells giving rise to hematopoietic progenitor. This pattern was consistent with previous reports[608,622,623], supporting the idea the SOX17 is down-regulated during EHT. Nevertheless, hematopoietic progenitors generated in this protocol, even if closely resemble their AGM-derived counterparts, failed to support long-term repopulation *in vivo*[597]. There is no reliable marker enabling an accurate separation of definitive AGM-like progenitors from primitive yolk sac-derived cells *in vi*tro[418,537]. Therefore, the potential of the *in vitro* generated progenitors has consistently been used to try to establish the primitive versus definitive origin of the PSC-derived cells. Based on this approach, many publications in the recent years reported the generation of progenitors defined as "definitive" based on their potential to generated T lymphocytes[467,539,569,597,624]. However, it is widely accepted that T lymphocyte emergence cannot be consider as a reliable indication of the definitive origin of the cells, since T and B lymphocytes arise *in vivo* during embryonic development prior AGM formation, suggesting that primitive lymphoid progenitors exists, and T cell potential cannot rule out primitive hematopoietic origin[410,413,414]. In fact, the only cell type that has been shown to originate exclusively from definitive HSCs is the mouse type2 B cells[413,417], an aspect that appeared to be conserved in humans as well[625,626]. So far, few protocols generating human B cells from PSCs have been reported[542], and in most of these cases the discrimination between the HSC-independent type1 and the HSC-dependent type2 B cells have not been assessed. More recently, the generation of mouse PSC-derived progenitors with the ability to engraft irradiated recipients and give rise to multilineage progeny, including type2 B cells, has been reported[609]. In this protocol, the combination of BMP4, FGF, Activin A and VEGF induced the differentiation of cKit+ cells with

repopulation potential when injected intra-femur into lethally irradiated NSG mice[609]. However, these progenitors displayed a limited self-renewal capacity and the same approach has not been tested with human PSCs.

2.8.4 Generation of human PSC-derived HSPCs: alternative approaches

Even if a broad spectrum of hematopoietic cells have been successfully differentiated from human and mouse PSCs, the generation of *bona fide* dHSCs is still an unachieved goal. Very few studies reported the generation of PSC-derived progenitors, which can successfully be transplanted into immunodeficient recipients, and even in these cases they only show a poor, short-term engraftment occasionally restricted to the myeloid lineage and secondary transplantation is rarely evaluated[465,627–629]. The successful generation of transplantable hematopoietic cells was achieved in mouse PSCs by over-expressing the transcription factor HOXB4[630,631] and by intra-femoral injection of cKit+ progenitors differentiated *in vitro* in a very limited window of time[609]. However, at least for the second approach, the observed engraftment was limited in time and no secondary transplantation potential was provided. For this reason, alternative approaches have been tested in the last decade, based on the forced expression of specific transcription factors to induce the reprogramming of somatic cells[632–634] or iPSC[635,636]. Moreover, based on the intrinsic ability of PSCs to differentiate in potentially all tissues of the body, the presence of hematopoietic cells within disorganised embryonic tissues (teratoma) generated by injecting human PSCs into immunodeficient mice have been evaluated[637,638] as a potential source of human PSC-derived HSCs.

2.8.4.1 Reprogramming strategies by transcription factor overexpression

The idea of using forced expression of transcription factors to reprogram the cell fate of different cell types to hematopoietic progenitors has been considered since the early nineties[639,640], but it is only after the advent of iPSC technology[603,641] that this sort of approach gained increasingly more interest. Several approaches have been tested both in human and mouse cells, ranging from the lineage conversion of adult somatic cells and iPSC, to the forced induction of EHT in endothelial cells (Table 1-8).

2.8.4.1.1 Lineage conversion from somatic cells or pluripotent stem cells (PSCs)

The feasibility of the lineage conversion approach applied to the hematopoietic system was first demonstrated by showing that the forced expression of RUNX1, HLF, LMO2, PRDM5, PBX1, ZFP37 in mouse lymphoid and myeloid committed progenitors was able to reprogram the cells into HSCs with multi-lineage engraftment potential in primary and secondary recipients[642]. Expression profile studies comparing PSC-derived hematopoietic progenitors with *ex vivo* isolated progenitors, highlighted the fact that the *in vitro* generated cells present higher levels of *HOXB* genes and lower expression of *HOXA* genes[578,643,644]. Based on these observations, the combined expression of transcription factors involved in hematopoietic differentiation has been tested in human PSCs. Elcheva et al. demonstrated that the overexpression of only two transcription factors could induce the differentiation to either endothelial or hematopoietic cells from PSCs. More precisely, the authors showed that the forced expression of ETV2 and ERG was able to drive the differentiation of endothelial cells. Conversely, ETV2 and GATA2 overexpression induced the emergence of CD43+ hematopoietic progenitors with pan-myeloid potential through an endothelial intermediate step. Moreover, the expression of GATA2 and TAL1 was enough to differentiate hematopoietic cells restricted to the erythroid and megakaryocytic lineages.

However, none of these progenitors were able to sustain long-term engraftment *in vivo*[544]. Doulatov and colleagues proposed an alternative approach by demonstrating that the forced expression of five transcription factors (HOXA9, ERG, RORA, SOX4 and MYB) in human iPSCs was sufficient to trigger the differentiation of CD34+ CD45+ CD38- hematopoietic progenitors capable of transient erythro-myeloid engraftment when injected *in vivo*[645]. More recently, the simultaneous expression of seven transcription factors (ERG, HOXA5, HOXA9, HOXA10, LCOR, RUNX1 and SPI1) in human iPSC-derived hemogenic endothelium was reported to be successful in generating hematopoietic progenitors with long-term repopulation ability[646]. Moreover, the overexpression of HOXA9 appeared to promote hematopoietic differentiation *in vitro*, even if no improvement was observed on the engraftment potential *in vivo*[644]. Likewise, the forced expression of HOX9, HOX7 and HOX5 did not show any effect on the repopulation ability of iPSC-derived progenitors[578]. Finally, despite the efficient induction of hematopoietic cells differentiation by the overexpression of HOXB4 in mouse PSCs[630], the same approach failed to generate hematopoietic progenitors from human iPSC[629]. Reprogramming strategies have been also applied to cells other then PSCs, such as human and mouse fibroblasts. A successful generation of hematopoietic progenitors capable of efficient myeloid engraftment was obtained by overexpressing OCT4 in human adult fibroblasts[647]. Moreover, the forced expression of GATA2, GFI1b, ETV6, c-FOS was able to induce a transcriptional hematopoietic program in mouse fibroblast[633], whereas the combination of ERG, GATA2, LMO2, RUNX1c, SCL expression enabled the differentiation of hematopoietic colony-forming cells with erythroid and megakaryocytic potential[632].

2.8.4.1.2 Induction of EHT in endothelial cells by TF overexpression

The use of endothelial cells (ECs) reprogramming to induce hemogenic endothelium conversion and hematopoietic differentiation avoiding a pluripotent intermediate step has been proposed as an alternative system. The forced expression of four transcription factors in combination with the exposure to an instructive vascular niche induced by the expression of adenoviral E4ORF1 gene in endothelial cells appeared to be a successful approach. Indeed, human umbilical vein endothelial cells (HUVEC) and mouse adult endothelial cells were efficiently converted into HSCs by the overexpression of FOSb, GFI1, RUNX1 and SPI1[634,648]. The generated EC-derived HSCs displayed multi-lineage long-term engraftment in both primary and secondary recipients as well as a transcriptome comparable to that of adult hematopoietic stem cells[648].

Table 1-8. Differentiation of human hematopoietic stem and progenitor cell: reprogramming strategies

Cell type	Hematopoietic induction	HSPCs phenotype	Transcription Factors	Differentiation potential	Engraftment in vivo	HE	Ref.
hFibroblasts	-		OCT4	Myeloid and erythroid	long-term myeloid		643
mFibroblasts	-	CD45+CD34+Sca1+cKit+CD48-CD150+ LT-HSC	GATA2, GFI1b, ETV6, c-FOS	n/a	n/a	Y	637
mFibroblasts (p53-/-)	-	CD41+cKit+ MPP / LSK HSCs	ERG, GATA2, LMO2, RUNX1c, SCL	Myeloid, erythroid, megakaryocytic, B and T lymphoid	short-term erythroid	Y	628
HUVEC/adult hECs	E4ORF1 niche	CD45-lin-CD38-CD34+CD45RA-CD90+/-	FOSb, GFI 1, RUNX1, SPI1 (constitutive)	CFU-GM, GEMM, E	long term CFU-GM, GEMM, E	-	630
mECs	E4ORF1 niche	Ve-Cad+Runx1+CD45+	FOSb, GFI 1, RUNX1, SPI1 (conditional)	CFU-GM, GEMM, E	long-term myeloid/lymphoid	-	644
mCommitted Progenitors	-	MEP, GMP, CMP, CLP, MkP, MPP, HSC	RUNX1, HLF, LMO2, PRDM5, PBX1, ZFP37	CFU-G, GM, M, GEMM and B cells	long term	-	638
hPSC-derived CD34+	EB	CD34+CD38-	HOXA5, HOXA7, HOXA9	n/a	no engraftment	n/a	575
hPSC	OP9/EB	CD34+CD45+	HOXA9	CFU-G, GM, M	no engraftment	n/a	640
hPSC	-	Ve-Cad+CD43+	GATA2, ETV2	Myeloid, erythroid and Megakaryocytic	no engraftment	n/a	541
hPSC	-	Ve-Cad+CD43+	GATA2, TAL1	Erythroid, megakaryocytic	no engraftment	n/a	541
hPSC-derived CD34+CD45+	EB	CD34+CD38-CD90+CD49f+	ERG, HOXA9, RORA, SOX4, MYB	CFU-G, M, GM, GEMM, E and T cells	short-term erythroid/myeloid	n/a	641
hPSC-derived HE	EB	n/a	ERG, HOXA5, HOXA9, HOXA10, LCOR, RUNX1, SPI1	Myeloid, erythroid B and T cells (*in vivo*)	long-term (secondary recipients)	Y	642

2.8.4.2 Human PSC-derived HSPCs in vivo: teratoma formation

Along with *in vitro* differentiation and reprogramming, an alternative approach to generate PSC-derived hematopoietic progenitors consists in the *in vivo* generation of teratomas. *In vivo* teratoma formation is achieved by injecting human PSCs into immunodeficient mice. Pluripotent cells give rise to disorganised tissues (teratoma) belonging to the three different germ layers, including structures resembling the bone marrow environment[637,649]. To date, two publications have tested the ability of human PSCs to differentiate in definitive HSCs by injecting them in NSG mice along with OP9-DLL1/WNT3a stromal cells[637] or by providing the human cytokines SCF and TPO[638]. As a result, human CD45+ cells were generated in the teratoma, which showed the ability to home to the bone marrow and seed other lymphoid organs through circulation[637,638]. Moreover, generated CD34+ CD45+ progenitors were capable of engrafting primary recipients up until 12 weeks, as well as secondary recipients for up to 4 weeks after transplantation[637]. Finally, teratoma-derived B and T cells appeared to be functionally normal and erythrocytes expressing adult globin could be detected. However, even if promising results were reported, a longer period of incubation is needed to clearly establish if those generated progenitors have the same long-term repopulation ability of *bona fide* dHSCs[426].

2.9 Human iPSCs as a source of functional immune cells

The differentiation of human hematopoietic cells from iPSC not only represents a very powerful approach to better understand the mechanisms involved in the early events of human embryonic haematopoiesis, but it also enables the opportunity to generate *in vitro* the whole spectrum of cells composing the human immune system. The potential applications for such a technology could cover a wide range of possibilities, varying

from modelling of haematological diseases to cell immunotherapy and drug discovery. Therefore, the establishment of reliable protocols to efficiently differentiate most of the hematopoietic lineage represents an important goal that has not been achieved yet, even though the successful generation of many types of hematopietic cells, such as T cells[650,651], B cells[652], NK cells[653], red blood cells[654,655] and myeloid cells[656] has been reported to date. However, the lack of an efficient approach to generate definitive human HSCs represents a fundamental limitation of this process, and an in-depth characterisation of the hematopoietic programs involved in the generation of the aforementioned cells is strongly required.

2.9.1 PSC-derived mononuclear phagocytes: successes and unachieved goals

Among all the immune cell types that have been successful generated from human PSCs to date, of particular interest for this project are the members of the mononuclear phagocyte system (MPS), such as monocytes, macrophages and DCs (Table 1-9).

2.9.1.1 Human PSC-derived monocytes

The differentiation of monocytes from human embryonic stem cells (ESC) or induced-pluripotent stem cells (iPSC) has been reported in several publications[657–660]. However, in most cases monocytes are generically described as CD45+ CD14+ cells that arise in the latest stages of PSC differentiation. Only few studies reported a more accurate characterisation of these cells[658,659], showing that they also express the chemokine receptor CX3CR1, the scavenger receptors CD36 and CD163, the cytokines receptors CD116 (GM-CSFR) and CD115 (M-CSFR) and the Fc receptors CD32 and CD64[658], all known to be expressed in human monocytes. However, these PSC-derived monocytic cells expressed low levels of CD62L and CCR2 as compared to monocyte

derived from adult CD34+ progenitors. Furthermore, these cells have been shown to migrate in response to the chemokine CX3CL1 and to produce IL1β when stimulated with LPS and ATP[659]. Finally, PSC-derived monocytes could be differentiated into moMacs and moDCs by exposing them to M-CSF and GM-CSF/IL-4, respectively[658,659].

2.9.1.2 Human PSC-derived macrophages

The generation of human PSC-derived macrophages has been reported in several publications[526,657–659,661,662], using both a feeder-free EB-based approach and OP9 co-cultures. However, only recently a more extensive analysis of the embryonic origin of those cells has been performed[661,662], revealing their primitive origin. Overall, the PSC-derived macrophages obtained with different protocols shared a consistent penothype, based on the expression of monocyte-macrophages markers such as CD14, CD16, CD163, CD115, CD11b and CD11c, among others[656–659]. Moreover, most of these protocols appeared to generated functional cells, as measured by their ability to efficiently take up antigens and they could also undergo polarisation towards the M1 or M2 phenotype, when exposed to IFN-γ and IL-4, respectively[658,659]. Nevertheless, all these aspects did not provide any information about the embryonic origin of these cells, since the terminally differentiated macrophages arising from primitive or definitive hematopoietic program are indistinguishable. Only recently two papers were published trying to identify the hematopoietic program involved in the generation of PSC-derived macrophages[661,662]. Buchriser et al. performed genetic experiments by knocking out transcription factors involved in the early steps of hematopoietic differentiation in the embryo using CRISPR/Cas9. This approached resulted in the characterisation of the CD14+ CD16lo CD163+ CD11b+ macrophages generated *in vitro* as MYB-

independent, RUNX1 and SPI1-dependent cells[661], demonstrating a primitive HSC-independent origin of those cells. At the same time, Takata and colleagues were able to monitor the differentiation of mouse and human iPSC-derived macrophages from *in vitro* generated EMPs through a hemangioblast intermediate step, supporting their primitive origin. More interestingly, mouse iPSC-derived macrophages had the ability to respond to environmental cues and to terminally differentiate *in vivo* in tissue resident macrophages, as demonstrated for alveolar macrophages and microglia[662]. Finally, transcriptome analysis of these cells confirmed their close relationship with yolk sac-derived macrophages, and highlighted significant differences when compared to bone marrow-derived ones[662].

2.9.1.3 Human PSC-derived dendritic cells (DCs)

In 2004 the generation of human ESC-derived antigen presenting cells was first reported. However, an extensive characterisation of the differentiated cells was not performed, and the exact identity of the HLA-DR+ leukocytes able to induce allogeneic T cells activation was not assessed[663]. In the following years, several other publications described the differentiation of human DC-like cells from both ESCs and iPSCs[656,659,664–666]. Most of these protocols were able to generated DC-like cells characterised by their ability to migrate, take up antigens and mature in response to activation stimuli such as LPS and TNFα, which were also able to trigger the production of pro-inflammatory cytokines. Moreover, their potential to induce allogeneic as well as antigen-specific autologous T cells proliferation was reported[656,659,664–666]. However, the extensive use of GM-CSF in the culture medium and the expression of monocytic markers such as CD14, CD11b, CD1a and CD209 strongly suggest that these PSC-derived cells closely resemble monocyte-derived DCs, and

therefore they do not represent a reliable model of human circulating dendritic cells[656,659,664,665]. Conversely, Silk et al. reported for the first time the generation of XCR1+ CD141+ human dendritic cells differentiated from iPSC[666]. The phenotype of these cells, even if not fully characterised, coincides with the human conventional cDC1 subset and this was further supported by their ability to efficiently cross-present tumor-associated antigens (TAA) and initiate an antigen-specific T cell response[666], the main functional feature of cDC1s. Finally, a recent publication described the generation of all human DC subsets (cDC1s, cDC2s and pDCs) from human iPSC in a two-step protocol[660]. A first differentiation of hematopoietic progenitors was performed in an EB-based protocol, and the generated progenitors, marked by the expression of CD31, CD43, and CD45, were subsequently differentiated to dendritic cells on OP9 feeders with the addition of the human recombinant cytokines GM-CSF, Flt3L and SCF. More interestingly, iPSCs knockout for the transcription factor IRF8 were generated, demonstrating that *in vitro* generated monocytes and DCs rely on IRF8 expression[660]. However, the phenotype of the PSC-derived DCs was not fully elucidated and the functional validation of the terminally differentiated cells was not provided, for instance by assessing their ability to present antigens or to produce subset-specific cytokines in response to TLR. Moreover, their dependency on IRF8, even if consistent with *in vivo* data from patients harbouring disease-causing mutations in IRF8[71], does not represent a strong validation of their identity, failing to exclude, for instance, a potential monocytic origin of these cells.

Table 1-9. *In vitro* differentiation of human MPS cells from iPSC.

Cell origin	Method	Cell product	Hematopoietic progenitors	Ref
hESC	EB	moDC	n/a	660
hiPSC	monolayer	macrophages	primitive/EMP	658
hiPSC	EB	CD141+XCR1+ DCs	n/a	662
hESC	OP9	moDC macrophages LC osteoclasts neutrophils eosinophils	CD34+CD43+ progenitors	652
hESC/hiPSC	monolayer	monocytes moDCs macrophages	n/a	655
hESC	EB	monocytes moDCs macrophages	n/a	654
hiPSC	EB	macrophages	primitive/EMP	657
hESC/hiPSC	EB	monocyte-macrophages osteoclast	n/a	653
hiPSC	EB	cDC1s cDC2s pDC s monocytes	CD34+CD43+ progenitors	656
hESC	EB	APCs	n/a	659
hESC/hiPSC	OP9	moDC	n/a	661

3 Overall aims and objectives

3.1 Rationale of the study

Due to their unique ability to efficiently process and present antigens to T lymphocytes, dendritic cells play a fundamental role in the establishment of a successful immune response. Most of the available knowledge about DCs development, regulation and function stem from genetic experiments performed *in vivo* in the mouse model. However, the paucity of easily accessible human DCs and the consequent inability to genetically manipulate these cells, has greatly limited the validation of these discoveries in the human system. Moreover, a better understating of the mechanisms driving an efficient immune response against cancer and the pivotal role of DCs in this context led to increased interest on their application in cell-based cancer immunotherapy[310]. However, even though numerous clinical trials have been performed using monocyte-derived DC this approach failed to provide a significant improvement in the overall clinical response in cancer patients[315,316], and evidences have been provided suggesting that the use of naturally circulating DC subsets may represent a more promising approach[315,321]. Hence, the establishment of a reliable system to generated DCs from induced-pluripotent stem cells may represent a major improvement both for the study and the potential clinical application of human dendritic cells. In this regard, several approaches have been proposed in the last decade[656,659,660,664–666], but the absence of a complete characterisation of the differentiated subsets and the lack of an exhaustive understanding of the embryonic hematopoietic program driving their differentiation *in vitro* greatly limited their application. Indeed, in order to achieve the successful differentiation of *bona fide* human DCs from iPSCs, it is essential to recapitulate the developmental processes leading to the generation of definitive hematopoietic progenitors during embryogenesis. Therefore, this project aimed at developing a new

approach to recapitulate *in vitro* and *in vivo* the development of human DCs from hematopoietic stem and progenitors cells. This knowledge will be practically implemented in the generation of DC subsets from human iPSCs. Once established, this approach may represent an invaluable tool to study human DC biology as well as their potential application in cellular immunotherapy, providing an unlimited source of patient-specific and genetically comparable cells, amenable to genetic manipulation.

3.2 Objectives

To this end, the following objectives will be pursued:

- to establish an experimental platform recapitulating DC differentiation from human hematopoietic stem and progenitor cells *in vitro* and *in vivo.*
- to promote the establishment of a definitive hematopoietic program in iPSC-derived progenitors enabling their terminal differentiation into functional human DCs;
- to generate genetically modified iPSC lines in order to study the transcriptional regulation of human DC development as well as manipulate their immunogenic function.

Chapter 2 Materials and Methods

1 Flow cytometry

1.1 Antibodies and reagents

Extracellular staining of cells was preformed in FACS buffer, consisting in PBS (GIBCO® Life Technologies) 1% BSA (Apollo Scientific) 2mM EDTA (Life Technologies). Antibodies used in all experiments are listed in Table 2-1.

Table 2-1. List of antibodies used in flow cytometry

Marker	Fluorochrome	Clone	Supplier
huCD45	APC-Cy7/APC	HI30	Biolegend
muCD45	PerCP-Cy5.5	104	eBiosciences
CD14	Pe-Cy7/BV786	HCD14/M5E2	Biolegend
CD16	BV421	3G8	Biolegend
CD141	PE/PE-Cy7	M80	Biolegend
CD1c	FITC/PE-Cy7	L161	Biolegend
Clec9A	PE/APC	8F9	Biolegend
HLA-DR	BV510	L243	Biolegend
CD123	PerCP-Cy5.5/APC	6H6	Biolegend
CD303	APC	201A	Biolegend
CD304	APC	12C2	Biolegend
CD163	FITC	GHI/61	Biolegend
CD206	PE/PerCP-Cy5.5	15.2/6H6	Biolegend
CD83	FITC	HB15E	Biolegend
CD86	PE	IT2.2	Biolegend
CD3	BV786	OKT3	Biolegend
Siglec6	FITC	767329	R&D
Axl	PE	108724	R&D
CD15	BV510	W6D3	Biolegend
CD19	PE	HIB19	Biolegend
CD34	APC	561	Biolegend
CD4	PerCP-Cy5.5	OKT4	Biolegend
CD8	APC-Cy7	HIT8A	Biolegend
PDL1	APC	29E.2A3	Biolegend
CD43	PE	1G10	BD Bioscience
CD73	APC-Cy7	AD2	Biolegend
CD90	BV510	5E10	Biolegend
CD41	FITC/PE-Cy7	A2A9/6	Biolegend
CD31	BV421	WM59	Biolegend
CD184	BV786	12G5	Biolegend
CD135	PE-Cy7	BV10A4H2	Biolegend

Marker	Fluorochrome	Clone	Supplier
CD45RA	BV510	HI100	Biolegend
α-tmSCF	biotin	polyclonal	R&D
α-Flt3L	biotin	polyclonal	R&D
CD2	PE	RPA-2.10	Biolegend
CD5	PE	L17F12	Biolegend
CD1a	APC	HI149	Biolegend
CD172a	PE	SE5A5	Biolegend
CD88	PE	S5/1	Biolegend
CD64	APC	10-1	Biolegend
CD11b	PE	ICRF44	Biolegend
CD33	APC	P67.6	Biolegend
NKp46	biotin	9E2	Biolegend
CD3	biotin	OKT3	Biolegend
CD19	biotin	HIB19	Biolegend
CD20	biotin	2H7	Biolegend
CD15	biotin	MC-480	Biolegend
CD203c	biotin	NP4D6	Biolegend
Streptavidin	APC-Cy7	-	Biolegend

1.2 Cell preparation and staining

Antibody staining for flow cytometry or fluorescence-activated cell sorting (FACS) was performed by incubating the cells for 20 minutes in FACS buffer containing the mix of antibodies at the appropriate concentrations. Samples were kept on ice and protected from light. After incubation, cells were washed twice in FACS buffer and analysed using BD LSR Fortessa or sorted on a BD FACS Aria running BD FACSDIVA (BD Biosceinces). DAPI 1μg/ml (PromoKine) was used for live/dead discrimination. Both instruments were housed at the Biomedical Research Centre (BRC) Flow Core Facility (Guy's and St Thomas' NHS Foundation Trust and King's College London). Analysis of flow cytometry data was performed using FlowJo software (TreeStar, version 10.2). Cell counts were obtained by adding 5μl/sample of AccuCheck beads (Thermo Fisher).

1.3 Intracellular staining

Intracellular staining for human Flt3 (CD135) was performed using BD Cytofix/Cytoperm Fixation kit (BD Biosciences) as per manufacturer's instructions.

2 Cell culture and primary cell isolation

2.1 Cell lines maintenance

Human iPSC lines iKCL4 and iKCL11 (courtesy of Dr. Dusko Ilic - KCL) were cultured in Matrigel-coated plates (BD Biosciences) in mTeSR1 medium (Stemcell Technologies) and maintained at 37°C 5% CO_2 5%O_2. Cells were washed with DMEM (Gibco® Life Technologies) and detached using PBS (Gibco® Life Technologies) 0.8mM EDTA (Gibco® Life Technologies) solution. When re-plated iPSC were incubated in medium supplemented with 10μM Rho-kinase inhibitor (Y-27632, - Santa Cruz Biotechnology) for 6-12 hours.

MS5 stromal cells were cultured in IMDM (Gibco® Life Technologies) supplemented with 10% heat-inactivated FBS (Gibco® Life Technologies), penicillin/streptomycin (Gibco® Life Technologies), 50μM β-mercaptoethanol (Gibco® Life Technologies) and maintained at 37°C 5% CO_2 20%O_2. Cells were washed with PBS and detached using a solution of PBS 0.25% trypsin (Gibco® Life Technologies) 0.5mM EDTA.

OP9 stromal cells were cultured in α-MEM (Gibco® Life Technologies) supplemented with 20% non heat-inactivated FBS, penicillin/streptomycin, 50μM β-mercaptoethanol and maintained at 37°C 5% CO_2 20%O_2. Cells were washed with PBS and detached using a solution of PBS 0.25% trypsin 0.5mM EDTA.

B16 mouse melanoma cells were cultures in RPMI (Gibco® Life Technologies) supplemented with 10% heat-inactivated FBS, penicillin/streptomycin, 50μM β-

mercaptoethanol and maintained at 37°C 5% CO_2 20%O_2. Cells were washed with PBS and detached using a solution of PBS 0.25% trypsin 0.5mM EDTA.

HEK 293T human embryonic kidney cells 293 were cultures in DMEM supplemented with 10% heat-inactivated FBS, penicillin/streptomycin, 50μM β-mercaptoethanol and maintained at 37°C 5% CO_2 20%O_2. Cells were washed with PBS and detached using a solution of PBS 0.25% trypsin 0.5mM EDTA.

SK-MEL28 human melanoma cells RPMI supplemented with 10% heat-inactivated FBS, penicillin/streptomycin, 50μM β-mercaptoethanol and maintained at 37°C 5% CO_2 20%O_2. Cells were washed with PBS and detached using a solution of PBS 0.25% trypsin 0.5mM EDTA.

2.2 Human iPSC co-cultures

For *in vitro* differentiation of human iPSCs, MS5 or OP9 cells were seeded in 6-well plates at a density of 10^5 cells/well and allowed to grow over confluent for 3-4 days. Medium was then changed to differentiation medium consisting in α-MEM supplemented with 10% not heat-inactivated FBS, penicillin/streptomycin, 100μM MTG (Sigma-Aldrich) and 10^5 iPSC/well were added. Every 3 days half of the medium was replaced with fresh differentiation medium. Cells were incubated at 37°C 5% CO_2 20%O_2. On the experiment end-point medium was collected in a 15ml Falcon tube and cells were incubated at 37°C for 30 minutes in 1mg/ml Collagenase D solution (Roche) in PBS. Supernatant was collected in the same 15ml Falcon tube and cells were incubated for 10 minutes at 37°C in 0.05% trypsin 0.5mM EDTA solution in PBS. Cells were collected, spun down, re-suspended in PBS and passed through a 100μm cell strainer (Corning). Cell suspension was then used in downstream applications.

2.3 Cord blood-derived CD34+ progenitors co-cultures

For *in vitro* differentiation of human HSPCs into DCs, MS5 or OP9 feeders were seeded in a 96-well plate (flat bottom) at a density of 10^4 cells/well. The day after, 10^4 cord blood-derived CD34+ cells/well were seeded on top of stromal cells in IMDM supplemented with 10% heat-inactivated FBS, penicillin/streptomycin, 50μM β-mercaptoethanol and maintained at 37°C 5% CO_2 20%O_2. Half of the medium was replaced at day 5 and 10, and cells were collected with a solution of PBS 5mM EDTA (at 4°C) at day 15 for flow cytometry analysis. For recombinant FSG samples, human Flt3L (Celldex) 100ng/ml, human SCF (Peprotech) 20ng/ml and human GM-CSF (Peprotech) 10ng/ml were added.

2.4 Isolation of human PBMC by Ficoll-Paque gradient

Human blood samples (both cord and peripheral blood) were diluted in warm PBS and layered on 15ml of Ficoll-Paque (GE Healthcare) in 50ml Falcon tubes. Tubes were than centrifuged for 30 minutes at 2000 rpm and 37°C without brake. The mononuclear cell layer was collected at the interphase and washed several times with warm PBS. Cells were then counted and used in down-stream applications.

2.5 Isolation of CD34+ cells by magnetic separation

PBMCs isolated from cord blood or cell suspensions from iPSC/OP9 co-cultures were incubated for 30 minutes at 4°C with CD34 Microbeads (Miltenyi) in FACS buffer according to manufacturer instructions. Cells were washed in FACS buffer and passed through a pre-washed MACS LS column (Miltenyi) placed in the magnetic field of a MACS separator. CD34+ cells retained in the column were washed three times with FACS buffer, the column was removed from the separator and cells were flushed into a new collection tube in FACS buffer. Purity was assessed by flow cytometry.

3 Molecular cloning

3.1 Cloning of human growth factors into retroviral vectors

Vectors, enzymes and primers for the generation of retroviral vectors for human growth factor expression are listed in Table 2-2. For all constructs, vectors were linearized by restriction enzymes digestion (New England Biolabs) (Table 2-2) and purified from agarose gel using QIAquick gel extraction kit (Qiagen) according to manufacturer's instructions. Human factors were amplified by PCR with specific set of primers using Q5 high-fidelity Taq Polymerase (New England Biolabs) (Table 2-3) and digested in order to create complementary overhangs for subsequent ligation into retroviral vectors. Digested amplicons were run on 1.5% agarose gel and purified as previously described. Linearized vector and insert were incubate at room temperature for 30 minutes in a reaction mix containing T4 DNA ligase (New England Biolabs) and provided buffer. Chemically competent bacteria DH-10 were transformed with the ligation product by heat-shock (30 minutes on ice, 30 seconds at 42°C, 5 minutes ice). Bacteria were then plated in LB agar plates containing 100μg/ml of Ampicillin at 37°C overnight. Selected colonies were expanded in LB at 37°C overnight and plasmid DNA was extracted using a Qiagen Miniprep kit (Qiagen). Successful ligation was assessed by Sanger sequencing.

Table 2-2. List of retroviral vectors used for human cytokines expression

Vector	Reporter	Selection	Restriction enzymes
pMX	-	Puromycin	BamHI/NotI
pMX IRES GFP	GFP	Puromycin	EcoRI/XhoI
pMX IRES mCherry	mCherry	Puromycin	EcoRI/XhoI

Table 2-3. Primers for the amplification of human growth factors

Cytokine	cDNA vector	Genebank ref	Primers
Flt3L	pCMV6-XL5	NM_001204502.1	GAATTCGCCACCATGACAGTGCTGGCGCC CTCGAGTCAGTGCTCCACAAGCAG
GM-CSF	pCMV6-XL4	NM_000758.3	GAATTCGCCACCATGTGGCTGCAGAGCCT CTCGAGTCACTCCTGGACTGGCTC
tmSCF	pCMV6-KITL	NM_003994.4	GGATCCGCCACCATGAAGAAGACACAAACTTGGA CTCGAGTTACACTTCTTGAAACTCTCTCTC
TPO	pMD-TPO	NM_000460.2	GAATTCGCCACCATGGAGCTGACTGAATTGC CTCGAGTTACCCTTCCTGAGACAGATTC

3.2 Cloning of sgRNA into CRISPR/Cas9 vectors

CRISPR/Cas9 vectors (Table 2-4) were digested with FastDigest BsmBI restriction enzyme (Life Technologies) in a 30 minutes reaction at 37°C. Linearized vectors were run on 1% agarose gel and purified using QIAquick gel extraction kit (Qiagen). Single guide RNA specific for each gene of interest (Table 2-5) were designed using an online tool from Zhang Lab (http://crispr.mit.edu). When possible, guides were directed to the first exon of the gene and high scored sequences based on the presence of off-targets were chosen. Oligos coding for sgRNA specific for each gene were annealed and phosphorylated by using the T4 PNK enzyme (New England Biolabs). The following parameters were used for successful annealing: 37°C for 30 minutes, 95°C for 5 minutes, rump down to 25°C at 5°C/min rate. Annealed oligos diluted 1/200 in sterile water were used. A ligation reaction with the linearized vector using T4 DNA ligase (New England Biolabs) was incubated for 30 minutes at room temperature. Chemically competent bacteria DH10 were transformed with the ligation product and plated on LB agar containing 100μg/ml Ampicillin overnight. Resulting colonies were amplified in LB at 37°C overnight and plasmid DNA was extracted using a Qiagen Miniprep kit (Qiagen). Successful ligation was assessed by Sanger sequencing.

Table 2-4. CRISPR/Cas9 lentiviral vectors

Vector	Selection	Restriction enzymes	Ref.
LentiCRISPR v1	Puromycin	BsmBI	[667]
LentiCRISPR v2	Puromycin	BsmBI	[668]
LentiGuide-Puro	Puromycin	BsmBI	[668]
LentiCas9-Blast	Blasticidin	BsmBI	[668]

Table 2-5. Single-guide RNA and validation primers for CRISPR/Cas9 targeting

Target gene	Single-guide RNA	Validation primers
Id2	CACCGGCTCATCGGGTCGTCCACA AAACTGTGGACGACCCGATGAGCC	AAGCTTTCGGGCTTCATTCTGAGC GAATTC TGCTGATATCCGTGTTGAGG
IRF8	CACCGTGGTCGGCGGCTTCGACAG AAACCTGTCGAAGCCGCCGACCAC	AAGCTTTTTCAGTTTGCACTCAGGGC GAATTCCGACCACAAAAGTGACTCCG
IRF4	CACCGCAAGCAGGACTACAACCGCG AAACCGCGGTTGTAGTCCTGCTTGC	AAGCTTTGCGTTACAGGAGAGCAGG GAATTCGCAAAACCCCTCAAGCCTTT
GATA2	CACCGCTTCAATCACCTCGACTCGC AAACGCGAGTCGAGGTGATTGAAGC	AAGCTTCCGCCTTCCTTTCGTTTTGA GAATTCAGTGGCATAGAAGGAACCCC
E2-2	CACCGCCCAACATTCCTGCATAGCC AAACGGCTATGCAGGAATGTTGGGC	AAGCTTCAGTTCAATTTTTACCAAGAAC GAATTCAGGCACAGAGCAAAACACAA
PDL1	CACCGTCTTTATATTCATGACCTAC AAACGTAGGTCATGAATATAAAGAC	GAATTCGCTTCTTTTGAGTGGGCAGA AAGCTTGCAACCAAGACTGAAAGATCA

4 Genetic modification by Lenti/Retroviral approaches

4.1 Lenti/Retrovirus production

A second-generation lentiviral packaging system psPAX2 (Gag/Pol/Rev) and pMD2.G (Envelop), and the retroviral packaging plasmid pCL-Ampho were used to produce viral particles for cells transduction. HEK-293T cells were seeded at a density of $3x10^6$ cells/plate in a 10-cm tissue culture dish and were co-transfected with lenti/retroviral vectors along with the corresponding packaging system using a calcium phosphate transfection method. Culture medium was re-placed 24 hours after transfection. Medium-containing virus particles was collected from transfected cultures after 48

hours of incubation, filtered through a 45μm syringe filter and used fresh or stored in 1ml aliquots at -80°C.

4.2 Lenti/Retrovirus transduction

All cell lines were transduced based on the following protocol. Cells were seeded in a 6-well plate the day before transduction in order to reach approximately 70% confluence within 24 hours. The following day the medium was removed and 2 ml of medium-containing virus were added along with 4μl of polybrene 4μg/μl (Sigma). Cells were centrifuged for 2 hours at 1600 rpm (spinoculation) and then incubated overnight at 37°C. The day after, medium-containing virus was removed and pre-warmed complete culture medium (specific for each cell line) was added to the plate. According to the selection marker carried by the lent/retroviral vector, cells were selected either by fluorescent activated cell sorting (FACS) or by antibiotic selection (concentrations specific for each cell line are reported in Table 2-6).

Table 2-6. Antibiotic concentrations for selection of transduced cell lines

Cell line	Puromycin (24hours)	Blasticidin (7 days)
iKCL4/iKCL11	0.5μg/ml	5μg/ml
MS5	15μg/ml	-
OP9	15μg/ml	-
SK-MEL28	0.5μg/ml	18μg/ml

5 Validation of CRISPR/Cas9 targeted cells

In order to validate the CRISPR/Cas9 targeting of human iPSC lines, genomic DNA was extracted from either monoclonal or polyclonal population of cells using GeneJET genomic purification kit (Thermo Fisher) according to manufacturer's instructions. The genomic region corresponding to approximately 600bp flanking the expected cut was

amplified by PCR using Q5 high-fidelity Taq Polymerase (New England Biolabs) with primers listed in Table 2-5. The resulting PCR products were purified from agarose gel and sent for sequencing (Eurofins Genomics). Sequencing results were analysed with the Track of Indels by Decomposition (TIDE) online tool [669].

When clones displaying heterozygous mutations were identified, the same PCR products were cloned into the vector pUC19 and competent bacteria were transformed (as described above). At least 10 independent colonies were analysed by Sanger sequencing, in order to identify the precise mutation characterising each allele.

6 Quantitative Polymerase Chain Reaction (qPCR)

Messenger RNA (mRNA) isolation was performed using RNeasy purification mini kit (Qiagen). Genomic DNA contaminations were eliminated from the samples by DNase I treatment (Roche) and mRNA was reverse transcribed into cDNA using a RevertAid RT kit (Thermo Fisher Scientific), according to manufacturer's instructions. Finally, the levels of expression of specific genes were evaluated by qPCR using the SensiMix SYBR kit (Bioline) and ABI Prism 7900HT (Applied Biosystems) analyzer. Relative quantification was performed using a ΔCt method, and raw data were normalized to an internal control (human GAPDH).

The following primers were use to detect the genes of interest:

Table 2-7. Primers for qPCR

GENE	Fwd Primer (5'-3')	Rev Primer (5'-3')
CEBPa	TGGACAAGAACAGCAACGAGTA	ATTGTCACTGGTCAGCTCCAG
PU1	GGAAGGGTTTCCCCTCGTC	GGTCGCTATGGCTCTCCCC
Pax5	TCCCAGCTTCCAGTCACAG	TGCTGCTGTGTGAACAAGTC
MPO	AGCGAGGAGCCCCTGGCCAGGAACCTG	GAGCTCGGGCATCTCACTGGAACGG
Id2	GCAGATCGCCCTGGACTCGC	AGCCACACAGTGCTTTGCTGTCA
IRF8	CTTCGACACCAGCCAGTTCTTC	ACAGCTCTTCCCAGCCTCTTCT
Flt3	CAAGTGCTGTGCATACAATTCCC	ACCTGTACCATCTGTAGCTGG
CXCR4	TATGACTCCATGAAGGAACCCTGT	AGCCTGTACTTGTCCGTCATGC

SOX17	CGCCGAGTTGAGCAAGAT	GGTGGTCCTGCATGTGCT
RUNX1	CTGCTCCGTGCTGCCTAC	AGCCATCACAGTGACCAGAGT
GATA2	CAAGATGAATGGGCAGAAC	CTTCTTCATGGTCAGTGGC
Ve-Cadherin	CAGCCCAAAGTGTGGAGAA	TGTGATGTTGGCCGTGTTAT
GATA1	CCAAGCTTCGTGGAACTCTC	ACTGACAATCAGGCGCTTCT
MYB	GCCAATTATCTCCCGAATCGA	ACCAACGTTTCGGACCGTA
NANOG	ACAACTGGCCGAAGAATAGCA	GGTTCCCAGTCGGGTTCAC
GAPDH	CAGCCTCAAGATCATCAGCA	GGGCCATCCACAGTCTTCT

7 Enzyme-Linked Immunosorbent Assay (ELISA)

In order to validate the generation of B16 melanoma lines expressing human Flt3L and human GM-CSF, the supernatant of cultured cells as well as the serum of tumor-bearing mice were analysed by ELISA. Protein expression was measured using a human Flt3L Quantikine Kit (R&D) and human GM-CSF ELISA MAX kit (Biologend) as per manufacturer's instructions.

8 Animal model (NSG)

NSG (NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) mice between 8 and 12 weeks old were used in all experiments. Animals were maintained under pathogen-free conditions in accordance with the UK Animal (Scientific Procedures) Act, 1986.

8.1 *In vivo* human DCs differentiation

Human cord blood-derived or iPSC-derived CD34+ hematopoietic cells were injected subcutaneously along with engineered stromal cells in 200µl of Matrigel (BD Biosciences). Mice were sacrificed at day 12 of differentiation by cervical dislocation and Matrigel plugs and, in some cases, spleens were collected.

In tumor experiments, B16 melanoma cells expressing human cytokines were injected intravenously (i.v.). At day 6 and 8, human PBMC were also injected i.v. and mice were sacrificed at day 10 by cervical dislocation. Lungs displaying tumor metastasis were collected, processed and analysed.

8.2 Preparation of cells from Matrigel plugs

Subcutaneous Matrigel plugs were recovered, cut in pieces and incubated in HBSS (GIBCO® Life Technologies) 1% FBS, 0.37U/ml Collagenase D (Roche), 10mg/μl DNaseI (Roche) and 1mg/ml Dispase (Sigma-Aldrich) for 30 minutes at 37°C. After digestion, plugs were smashed on a 100μm strainer (Corning) and cells were collected, spun down at 1600rmp for 5 minutes and re-suspended in FACS buffer for flow cytometry analysis.

8.3 Preparation of cells from mouse spleens

Mouse spleens were collected, cut in small pieces and incubated for 20 minutes at 37°C in digestion buffer, consisting of HBSS (GIBCO® Life Technologies) 1% FBS, 0.37U/ml Collagenase D (Roche) and 10mg/μl DNaseI (Roche). The reaction was stopped by transferring the samples on ice and adding EDTA at a final concentration of 2mM. Spleens were then smashed on a 100μm strainer (Corning) and the resulting cells suspension was spun down at 1600 rpm for 5 minutes. Red blood cells were then lysed with Ack lysis buffer (Life Technologies) for 5 minutes at room temperature and samples were spun down and re-suspended in FACS buffer for flow cytometry analysis.

8.4 Preparation of cells from lung metastasis

Lungs were harvested and transferred in a solution of 0.4mg/ml Collagenase IV (Sigma-Aldrich) in HBSS. Lugs were cut in small pieces and incubated at 37°C for 45 minutes. Resulting cell suspension was homogenized using a syringe with a 19G needle and

filtered through a 70μm cells strainer (Corning). Cells were then centrifuged for 5 minutes at 1400rpm 4°C and re-suspended in FACS buffer for flow cytometry analysis.

Chapter 3 Modelling human dendritic cells development

1 Introduction and objectives

Dendritic cells (DCs) are key effectors of the immune system for their central role in the initiation of antigen-specific immunity and tolerance. Dendritic cells have the unique ability to sense exogenous antigens and make them "visible" to T cells, promoting the initiation of a specific immune response and acting as a bridge between innate and adaptive immunity. Human DCs are a very rare population of circulating cells in the blood, and the paucity of easily accessible cells has represented a major limitation for their study. Therefore, the differentiation of human DCs, *in vitro* and *in vivo,* represents a fundamental tool to better understand their development and function. Moreover, of specific interest for this project, the generation of a reliable platform to induce the differentiation of HSPCs into human DCs can also be translated to iPSC-derived progenitors, to assess their differentiation potential and to open up new avenues for the study of human DCs in genetically tractable settings.

DC progenitors reside in the BM niche, and therefore the recapitulation of the niche microenvironment may represent an efficient approach to model DC development *in vitro*. To date, all the available protocols to differentiate human DCs are based on the use of GM-CSF[203]. However, experimental evidences from the mouse model demonstrated that GM-CSF is dispensable for DCs development *in vivo*[205]. Moreover, GM-CSF has been reported to strongly promote the differentiation of monocyte-derived cells *in vitro*[33,198] and *in vivo*[50,163,166], which only phenotypically resemble conventional DCs.

In vivo generation of human DCs has been achieved by treating fully reconstituted humanised mice with high doses of human Flt3L[66,70,179]. However, the establishment of a humanised model presents some technical limitations that can affect the utilisation of

this approach, such as the exposure of the animals to radiations as well as the time consuming process of successfully generating a fully reconstituted animal.

Therefore, the aim of this chapter was the establishment of an alternative approach to generate human DCs from HSPCs both *in vitro* and *in vivo*, by recapitulating the bone marrow niche microenvironment physiologically supporting DC development.

1.1 Objectives

In order to achieve this goal the following objectives were pursued:

- to generate a "humanised" stromal niche supporting human DC development, mimicking the bone marrow microenvironment;
- to identify the optimal combination of niche factors for human DC subset generation *in vitro* from hematopoietic progenitor cells;
- to investigate the heterogeneity of *in vitro* generated DC2s and the contribution of GM-CSF to their development;
- to improve human DCs differentiation *in vivo* by recreating an artificial niche-like environment in immunodeficient mice;

2 Results

2.1 MS5 stromal cells expressing human Flt3L are more efficient than OP9 in supporting human DC differentiation

In physiological conditions, human DCs are constantly replenished by progenitors residing in the hematopoietic niche of the bone marrow. Therefore, recreating a niche-like microenvironment *in vitro* may represent an efficient approach to expand dendritic cell progenitors and subsequently induce their differentiation into fully functional DC subsets. To test this hypothesis, an experimental strategy was developed, based on the

generation of an *in vitro* "humanised" niche by expressing human growth factors involved in hematopoietic progenitor maintenance as well as DC differentiation in bone marrow-derived murine stromal cell. Four factors were identified as potential candidates, including human Flt3L, CXCL12, SCF and TPO. Flt3L was chosen due to its fundamental role in DC development both *in vitro* and *in vivo*. CXCL12, SCF and TPO were selected based on mouse genetic evidences demonstrating their essential role in the maintenance and expansion of hematopoietic progenitors in the bone marrow niche. The genomic sequences coding for all these factors were amplified by PCR and subsequently cloned into retroviral vectors (pMX) carrying a selection marker (GFP, mCherry and PuroR, i.e.) downstream an internal ribosome entry site (IRES). Flt3L was cloned into pMX-IRES-GFP vector, TPO was cloned into pMX-IRES-mCherry and SCF was clones into a pMX vector not expressing any reporter gene. Finally an already available pBABE-Puro vector carrying the genomic sequence of human CXCL12 was used (Figure S3-1).

Two different mouse bone marrow-derived stromal cells lines were initially compared, MS5 and OP9. Both lines were transduced with retroviral vectors to constitutively express human Flt3L (MS5_Flt3L and OP9_Flt3L) and their ability to support human DCs differentiation was tested in a 15 days co-culture with cord blood-derived CD34+ hematopoietic stem and progenitor cells (HSPCs). Flow cytometry analysis of the terminally differentiated cells from three independent cord blood donors demonstrated the superior ability of MS5_Flt3L to support DCs differentiation, as expressed by the significantly increased number of CD141+Clec9A+, CD14-CD1c+ and CD123+CD303/4+ cells generated (Figure 3-1a). Furthermore, to test the advantage of generating stromal cells constitutively expressing the membrane bound form of Flt3L, the ability of MS5_Flt3L to induce DCs differentiation from cord blood-derived CD34+

HSPCs was compared with MS5 supplemented with recombinant Flt3L (100 ng/ml). Flow cytometry analysis of DC subsets at day 15 resulted in a significant increase of the frequency of CD141+Clec9A+ and CD14-CD1c+ cells in the total CD45+ population and consistent but not statistically significant increase of the total number of generated DCs. Conversely, pDC were efficiently generated in both systems (Figure 3-1b). Based on these observations, MS5 were subsequently used to test the ability of the identified growth factors to improve DCs differentiation. Therefore, seventeen lines expressing all the combinations of the four growth factors Flt3L, CXCL12, SCF and TPO were generated by retroviral transduction (Figure S3-1d). CXCL12-expressing lines were first generated and selected based on their resistance to Puromycin and the expression of human CXCL12 mRNA was confirmed by qPCR (Figure S3-1c). The validation of the FACS-sorted engineered lines was performed by flow cytometry by assessing the expression of fluorescence reporters (GFP and mCherry, i.e.) as well as trans-membrane proteins stained with antibodies (Flt3L and SCF, i.e.).

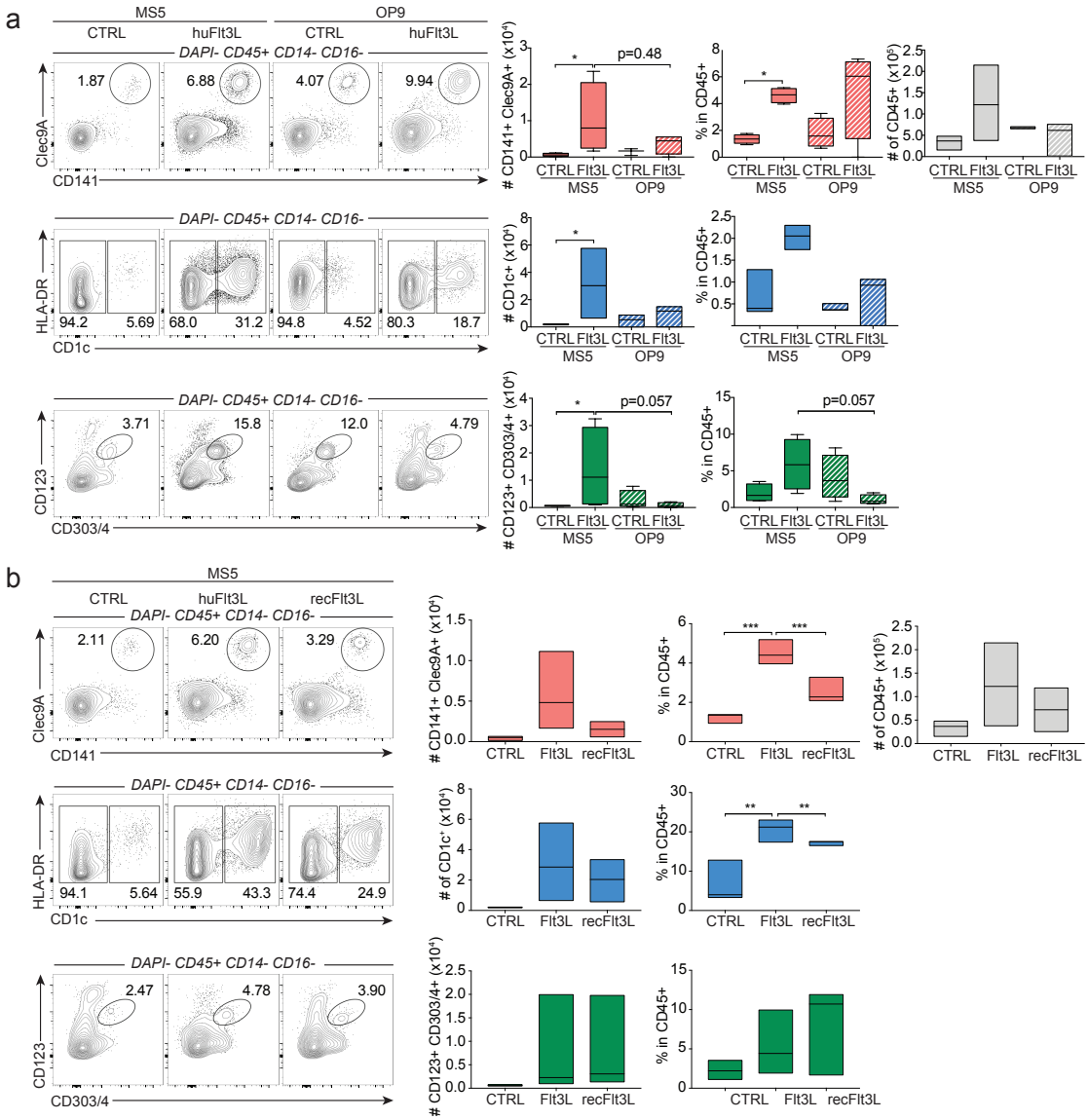


Figure 3-1. MS5 stromal cells expressing human Flt3L support DCs differentiation more efficiently than OP9. (a) Flow cytometry plots and quantification of human DC subsets differentiated *in vitro* from CD34+ cord blood-derived hematopoietic progenitors cultured with mouse stromal cell lines MS5 and OP9 expressing human Flt3L (MS5_Flt3L and OP9_Flt3L) at day 15. **(b)** Flow cytometry analysis at day 15 of CD34+ progenitors cultured with MS5 expressing human Flt3L or MS5 supplemented with recombinant human Flt3L. Summary of n=3 independent donors (mean ± SEM; * p<0.05, **p<0.01, ***p<0.001, Mann-Whitney U test).

2.2 Human DC differentiation strictly depends on Flt3L and it is improved by SCF and CXCL12

In order to identify the best combination of factors supporting human DCs differentiation *in vitro*, cord blood-derived HSPCs from three independent donors were seeded on the generated engineered MS5 lines and co-cultured for 15 days. Terminally differentiated cells were analysed by flow cytometry and the presence of CD141+Clec9A+, CD14-CD1c+ and CD123+CD303/4+ cells was assessed.

As expected, the presence of Flt3L was essential for DCs differentiation, as demonstrated by the absence of cells expressing DC-specific markers in all the conditions lacking Flt3L (Figure S3-2). Moreover, statistical analysis comparing the absolute number of cells as well as their frequency within the CD45+ gate resulted in the identification of MS5 expressing Flt3L, SCF and CXCL12 (MS5_FS12) as the best condition to generate both CD141+Clec9A+ and CD123+CD303/4+ cells. On the contrary, neither the numbers nor the frequency of CD14-CD1c+ cells was significantly different in all the tested conditions (Figure 3-2). On the other hand, a consistent and statistically significant reduction of both CD141+Clec9A+ and CD123+CD303/4+ cells was observed in all the condition expressing TPO, suggesting its inhibitory effect on the development of the two subsets. Conversely, the number and frequency of CD14-CD1c+ cells were not affected by the presence of human TPO (Figure 3-2). In conclusion, while the differentiation of CD141+Clec9A+ and CD123+CD303/4+ cells was modulated by the same combination of factors, CD14-CD1c+ cells development appeared to be unaffected in the same experimental conditions, in apparent contrast with the other two subsets.

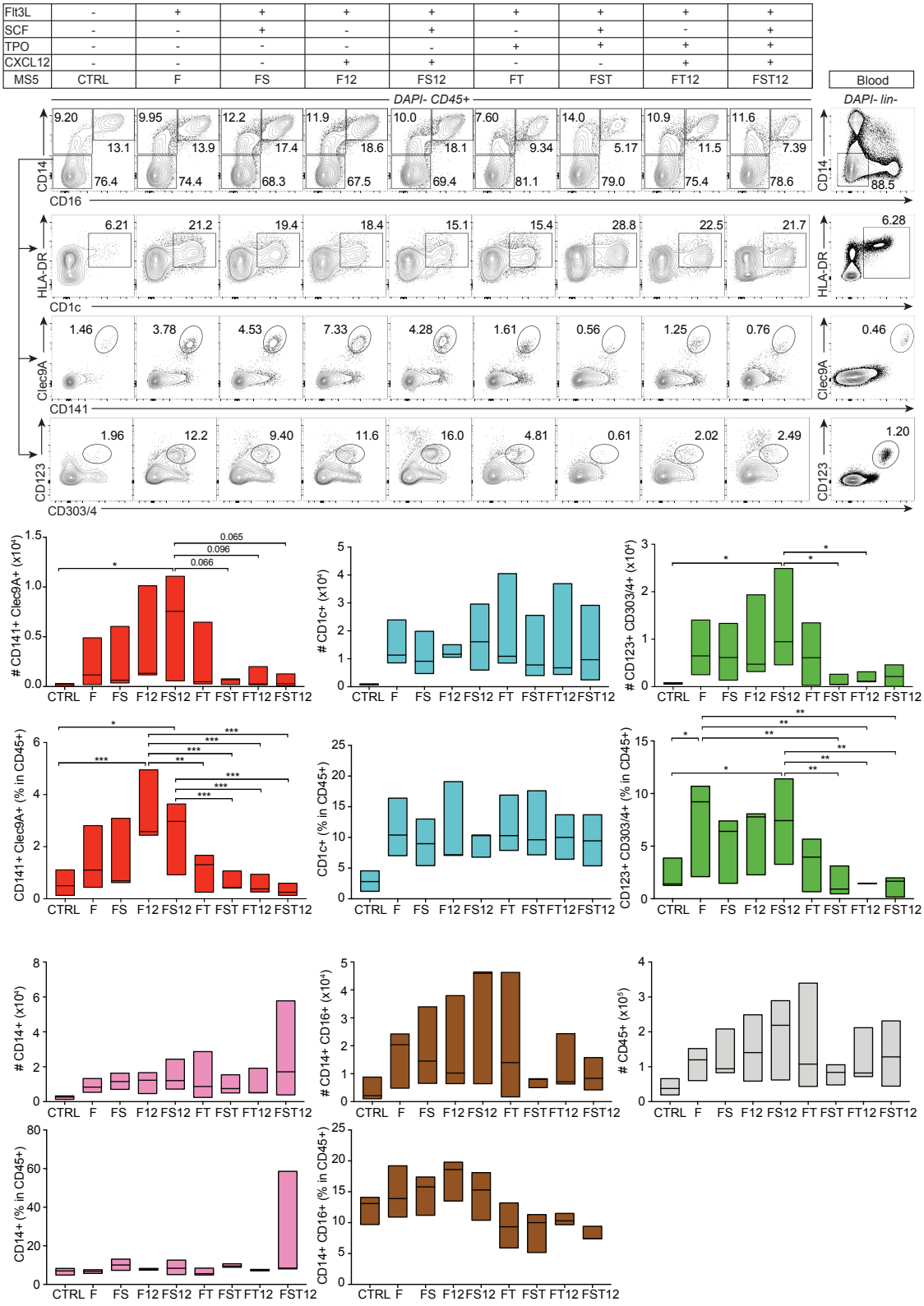


Figure 3-2. Human DC differentiation strictly depends on Flt3L and it is improved by the presence of the niche factors SCF and CXCL12. Representative flow cytometry plots assessing the potential of huFlt3L-expressing stromal lines to support DCs differentiation from cord blood-derived CD34+ progenitors in vitro. Quantification of differentiated cells from n=3 independent cord blood donors (mean ± SEM; * p<0.05, one-way ANOVA test).

2.3 *In vitro* differentiated CD1c+ cells are heterogeneous

In order to investigate the different regulation observed for CD1c+ cell development as compare to CD141+Clec9A+ and CD123+CD303/4+ subsets, a more extensive characterisation of these population was performed.

Further phenotypic analysis of the *in vitro* generated cells revealed the presence of two populations of cells within the CD14-CD1c+ subset, which can be separated based on the expression of CD206. More interestingly, while CD1c+CD206- cells were significantly increased in the MS5_FS12 condition, in accordance with the CD141+Clec9A+ and CD123+CD303/4+ subsets, the development of CD1c+CD206+ cells appeared to be promoted by the presence of TPO (Figure 3-3a and Figure S3-3a). In order to characterise the different populations of CD1c+ cells, the phenotype of cord blood-derived HSPCs differentiated *in vitro* on MS5_FS12 was compare to blood DCs by flow cytometry. Three subsets of CD1c+ cells were identified in the *in vitro* generated cells: the previously described CD14-CD1c+CD206- and CD14-CD1c+CD206+ cells along with a third subset of CD14+CD1c+CD206+ cells. On the contrary, only the CD14-CD1c+CD206- cells were detected in PBMC samples, corresponding to the human DC2 subset (Figure 3-3b). A more extensive analysis of the phenotype of these cells was also performed (Figure S3-3b) and the hierarchical clustering of mean fluorescence intensity (MFI) values of the selected markers measured by flow cytometry suggested a closer relationship of *in vitro* generated CD14-CD1c+CD206- and CD14-CD1c+CD206+ cells with blood DC2. In addition, CD14+ cells generated *in vitro* consistently aligned with human blood CD14+ monocytes (Figure 3-3c). To further characterise the different population of CD1c+ obtained in MS5_FS12 cultures, the ability of *in vitro* generated cells to respond to TLR stimulation was assessed by measuring the expression of the maturation markers HLA-DR, CD83

and CD86 by flow cytometry. When incubated overnight in the presence of LPS (TLR4), all the described CD1c+ subsets were able to significantly up-regulate the expression of HLA-DR, CD83 and CD86 (Figure 3-3d). Moreover, a preliminary experiment (n=1) using the TLR8 agonist VTX-2337 suggested that both CD14-CD1c+CD206- and CD14-CD1c+CD206+, but not CD14+CD1c+CD206+ and CD14+ cells, are equipped to respond to TLR8 stimulation (Figure 3-3d). Overall these experiments revealed the existence of two different subsets within the *in vitro* differentiated CD14-CD1c+ cells, which closely align to CD14-CD1c+CD206- blood DC2s based on their immunophenotype and their expression of functional TLR4 and TLR8. Moreover, the differentiation of CD14-CD1c+CD206- cells, a phenotype closely resembling blood *bone fide* DC2s, was significantly increased in MS5_FS12, further underling the superiority of this combination of factors in supporting human DCs differentiation.

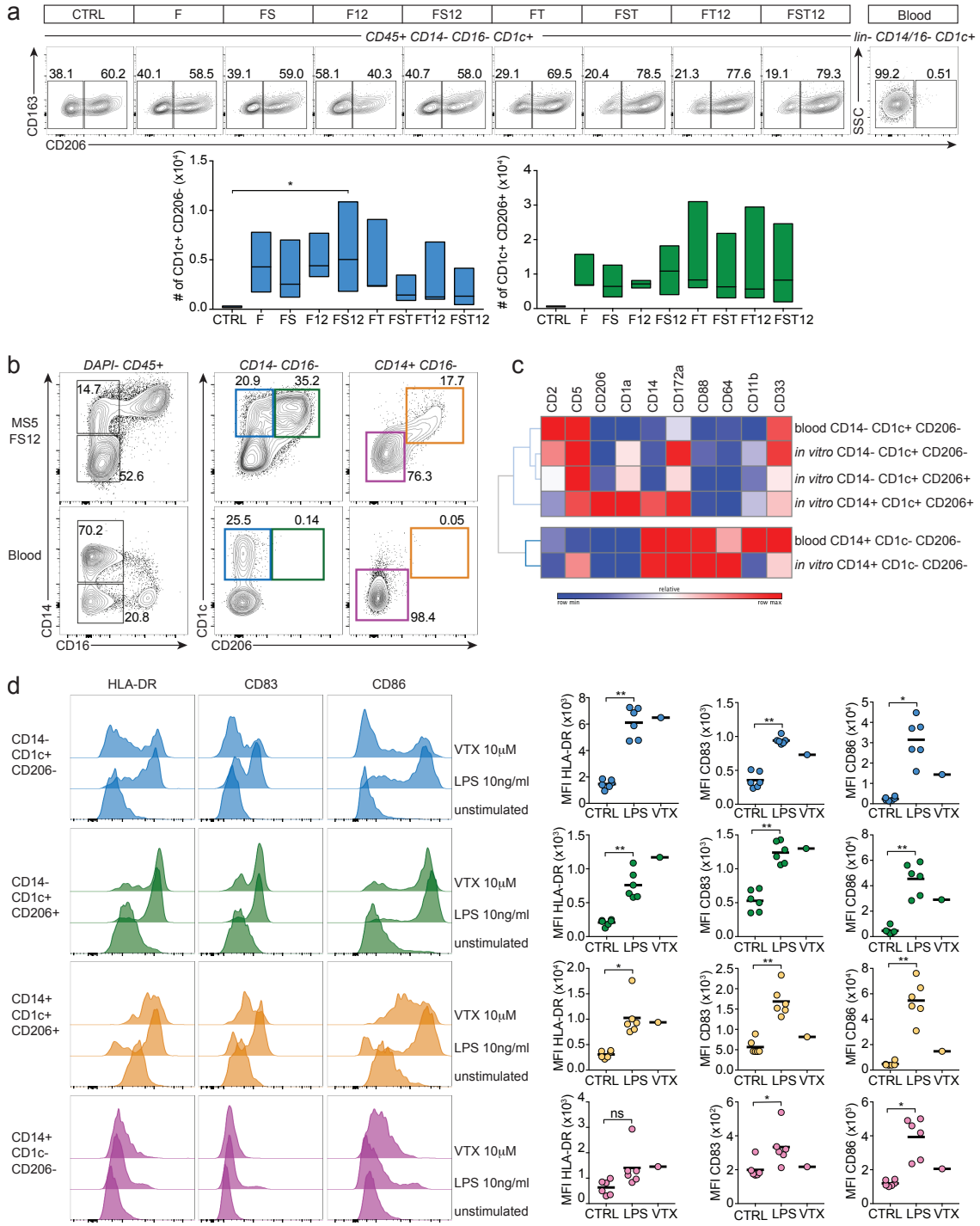


Figure 3-3. *In vitro* differentiated CD1c$^+$ cells are heterogeneous. (a) Representative flow cytometry plots and quantification of n=3 independent donors assessing CD1c$^+$ cells heterogeneity in CD34$^+$ progenitors co-cultures with engineered MS5 (mean ± SEM; * p<0.05, one-way ANOVA test). **(b)** Phenotype comparison of *in vitro* generated CD1c$^+$ cells (MS5_FS12) with their *ex vivo* isolated counterparts (blood). **(c)** Hierarchical clustering of flow cytometry data comparing mean fluorescence intensity (MFI) values from *in vitro* and *ex vivo* CD1c$^+$ cells using GENE-E analysis platform (Broad Institute) (mean of n=4 independent cord blood and PBMC donors). **(d)** Assessment of *in vitro* generated DCs maturation in response to TLR4 (LPS) and TLR8 (VTX) agonists by measuring the up-regulation of the activation markers CD86, CD83 and HLA-DR by flow cytometry (n=5 for LPS and n=1 for VTX. Mean ± SEM; * p<0.05, ** p<0.01, *** p<0.001, one-way ANOVA test).

2.4 CD14-CD1c+CD206- cells differentiated *in vitro* cannot be generated using GM-CSF alone

Previous experiments demonstrated how CD14-CD1c+CD206- and CD14-CD1c+CD206+ cells differentiated *in vitro* using MS5_FS12 stromal cells share several features, such as their dependency on Flt3L (Figure 3-2 and Figure S3-2), their phenotypic proximity to circulating blood DCs (Figure 3-3c) and their responsiveness to TLR4 and TLR8 (Figure 3-3d). Therefore, to further characterise the *in vitro* generated CD14-CD1c+CD206- and CD14-CD1c+CD206+ cells, their dependency on human GM-CSF, a cytokine extensively used to generate human DCs and moDCs *in vitro*, was assessed.

MS5 stromal cells expressing human GM-CSF (MS5_GM) were generated by retroviral transduction (Figure S3-1b) and their ability to induce DCs differentiation was compared to MS5_FS12 in 15 days co-cultures of cord blood-derived CD34+ progenitors.

Unlike the other CD1c+ subsets generated *in vitro*, CD14-CD1c+CD206- cells strictly require Flt3L for their generation and cannot be detected in presence of GM-CSF only (Figure 3-4a-b). Conversely, CD14-CD1c+CD206+ and CD14+CD1c+CD206+ cells can be generated in both conditions (MS5_FS12 and MS5_GM), and appeared to be induced more efficiently in MS5_GM, even though the observed difference did not reach statistical significance (Figure 3-4a-b). Moreover, two additional populations of CD1c- cells were observed exclusively in presence of GM-CSF: the CD14-CD1c-CD206+ and the CD14+CD1c-CD206+ subsets (Figure 3-4a). More interestingly, the induction of these populations by GM-CSF correlates with a statistical significant decrease of CD14+CD1c-CD206- monocytes in MS5_GM samples (Figure 3-4b).

In conclusion, even if all the *in vitro* generated CD1c+ cells depend on Flt3L for their differentiation (Figure 3-2 and Figure S3-2), the CD14-CD1c+CD206+ and

CD14+CD1c+CD206+ subsets can also be induced by using GM-CSF alone. Conversely, CD14-CD1c+CD206- cells exclusively rely on Flt3L for their development.

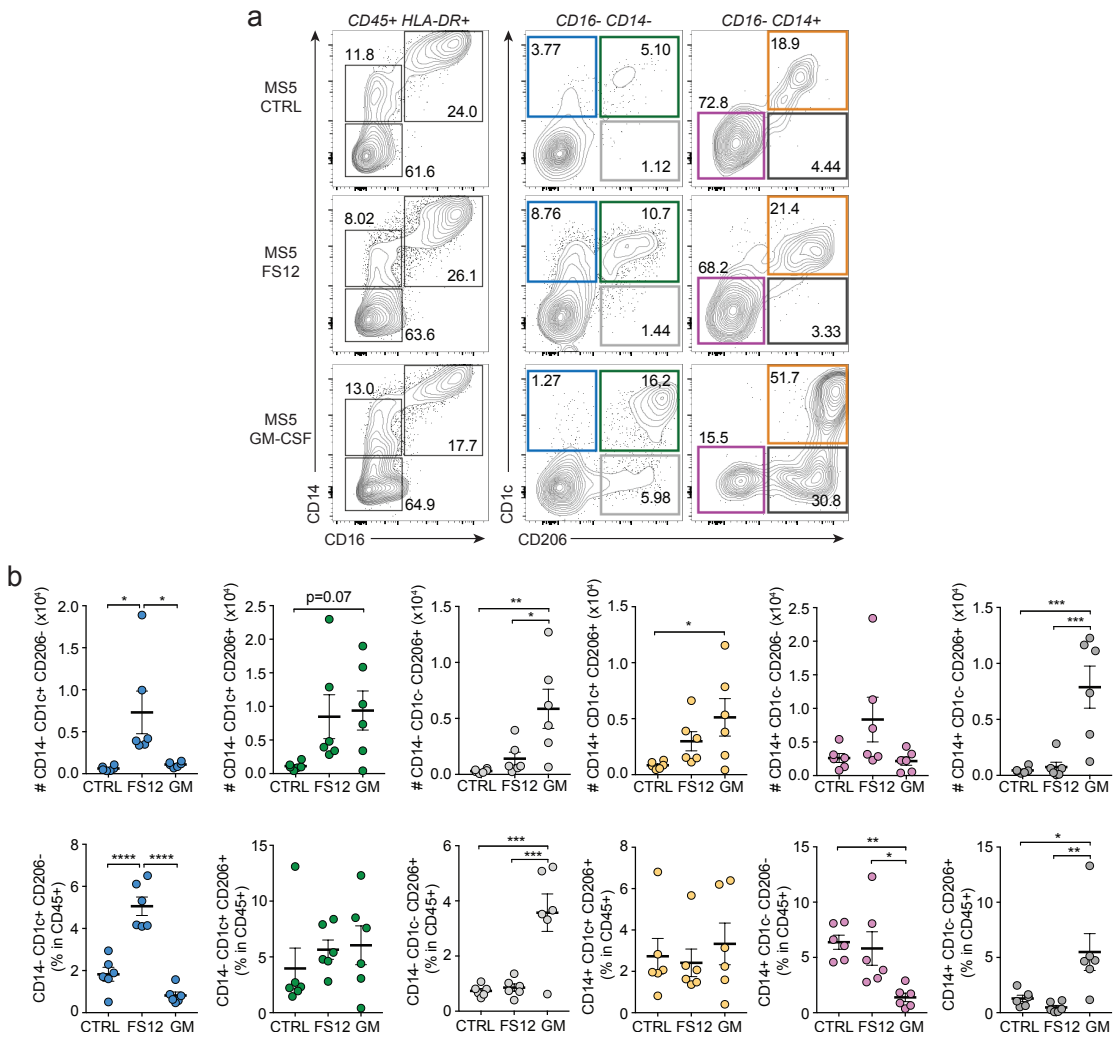


Figure 3-4. MS5_FS12 support the differentiation of CD1c$^+$ CD206$^-$ cells that cannot be generated with GM-CSF alone *in vitro*. (a) Representative flow cytometry plots and quantification of CD34$^+$ progenitors co-cultured with MS5, MS5 expressing human Flt3L, SCF and CXCL12 (MS5_FS12) and MS5 expressing human GM-CSF for 15 days. **(b)** Quantification of differentiated cells from n=5 independent cord blood donors in 3 independent experiments (mean ± SEM; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 one-way ANOVA test).

2.5 MS5_FS12 are superior to MS5+recombinant FSG to support human DC differentiation *in vitro*

The most commonly used protocol to differentiate human DCs *in vitro* from hematopoietic progenitors is based on the co-culture of CD34+ HSPCs with Mitomycin C-treated MS5 supplemented with the recombinant human cytokines Flt3L, SCF and GM-CSF (recFSG) [203]. In order to compare the efficiency of this approach with the MS5_FS12 stromal cells generated, the two systems were tested in a 15 days co-culture experiment using cord blood-derived CD34+ progenitors. Flow cytometry analysis of the cells generated at day 15 highlighted the superiority of MS5_FS12 in supporting the differentiation of CD141+Clec9A+, CD14-CD1c+CD206- and CD14-CD1c+CD206+ cells, both in absolute numbers and frequency of CD45+ cells (Figure 3-5a and Figure S3-5). Moreover, a major phenotypic difference was observed for CD141+Clec9A+ cells differentiated in MS5_FS12, which, unlike their recFSG counterparts, lacked the expression of CD1c, more closely resembling the phenotype of circulating blood DC1 (Figure 3-5a). Furthermore, based on the total number of cells and their frequency within the CD45+ population, MS5_FS12 appeared to be more efficient in generating CD123+CD303/4+ cells and they were also capable of giving rise to a very rare population of Axl+Siglec6+ cells (Figure 3-5b). Overall, MS5_FS12 yielded significantly higher numbers of CD45+ hematopoietic cells as well as CD15+ granulocytes, even though CD15+ cells appeared to be significantly more frequent in recFSG samples (Figure 3-5c and Figure S3-5). Finally, CD3+ T cells and CD19+ B cells were not detected in both approaches, but MS5_FS12 were capable to maintain a significantly larger population of undifferentiated CD34+ cells as late as day 15 (Figure 3-5c). Therefore, based on their ability of generating larger number of all the analysed subsets, as well as providing a more accurate phenotype for CD141+Clec9A+ cells, MS5_FS12 resulted to be a better system to support human DC differentiation *in vitro*.

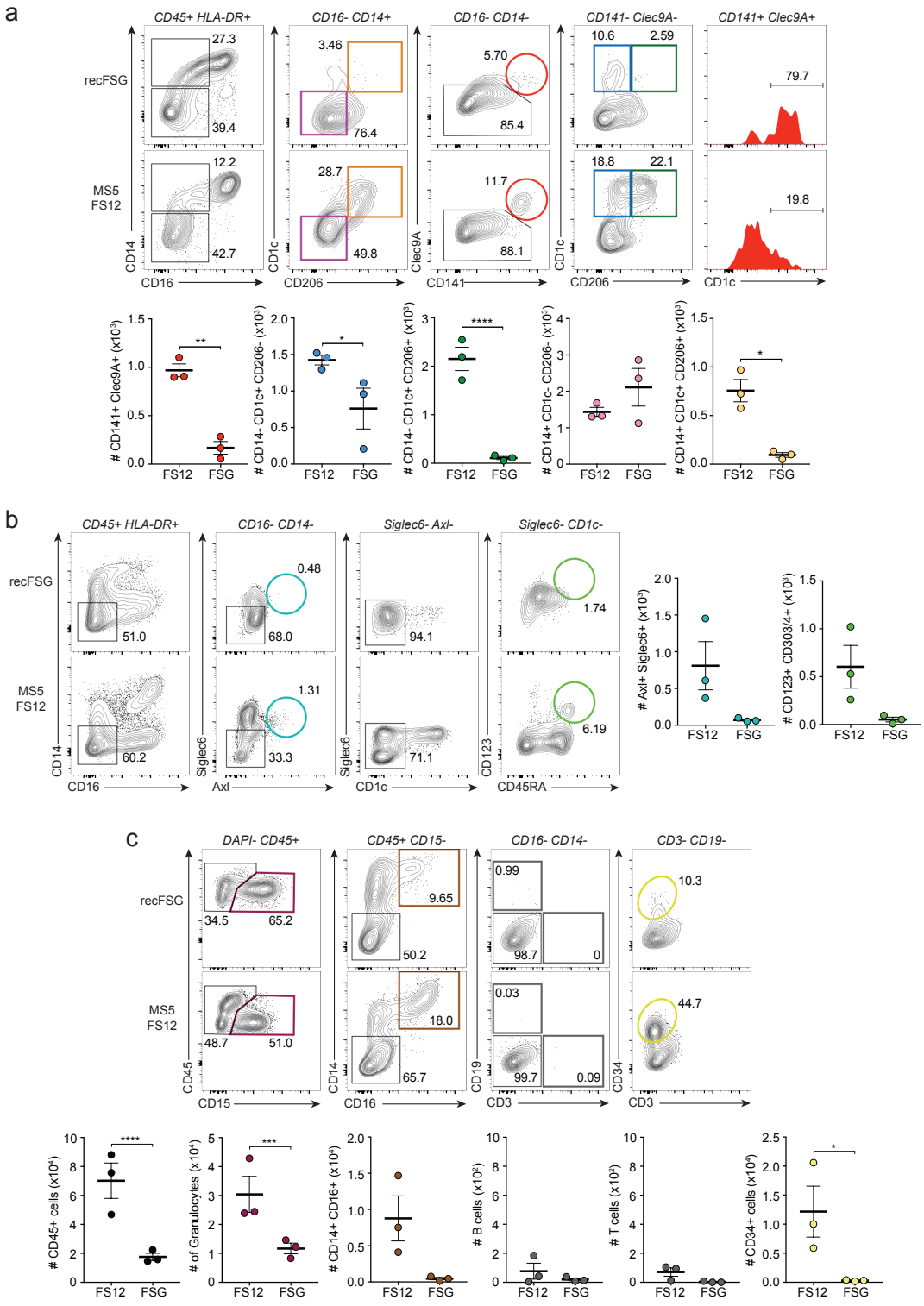


Figure 3-5. MS5_FS12 are superior to recombinant FSG to support human DCs differentiation from cord blood-derived hematopoietic progenitors. Flow cytometry analysis and quantification of human monocytes, conventional and plasmacytoid DCs **(a-b)** and other leukocytes **(c)** generated from $CD34^+$ progenitors seeded on MS5 expressing human Flt3L, SCF and CXCL12 (FS12), as compared to mitomycin-treated MS5 supplemented with recombinant Flt3L, SCF and GM-CSF (FSG) (n=3 independent cord blood donors. Mean ± SEM; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ two-way ANOVA test).

2.6 MS5_FS12 promote human DCs differentiation *in vivo* in subcutaneous implants in NSG mice

In order to test the ability of MS5_FS12 stromal cells to support human DCs differentiation *in vivo*, cord blood-derived CD34+ progenitors were injected along with MS5_FS12 or MS5_CTRL in subcutaneous Matrigel-based implants. The use of synthetic basement membrane matrix (Matrigel) allowed the generation of "organoids" localised under the skin of immunodeficient mice retaining both the supportive stromal cells and the CD34+ progenitors in close contact and favouring their interaction. After 12 days of differentiation, the Matrigel-based "organoids" were recovered and their cellular content was analysed by flow cytometry. Human CD45+ hematopoietic cells were detected in both MS5_FS12 and MS5_CTRL, even if significantly higher numbers of cells were obtained from the stromal cells expressing human cytokines (Figure 3-6). In addition, unlike MS5_CTRL, MS5_FS12 were capable of supporting the differentiation of CD141+Clec9A+ and CD14-CD1c+ cells. On the other hand, CD123+CD303/4+ cells were generated in both conditions (MS5_CTRL and MS5_FS12) at the same frequency within CD45+ cells, even though MS5_FS12 yielded a significantly higher amount of cells (Figure 3-6). Furthermore, when compare to *in vitro* cultures, MS5_FS12 failed to give rise to CD14-CD1c+CD206+ and CD14+CD16+ cells, even though a not significant increase in the number of CD14+CD1c-CD206- and CD14+CD1c+CD206+ cells was detected (Figure 3-6).

All together these results demonstrated that the MS5_FS12 stromal niche was capable of supporting the differentiation of CD141+Clec9A+, CD14-CD1c+ and CD123+CD303/4+ cells *in vivo* in immunodeficient NSG mice.

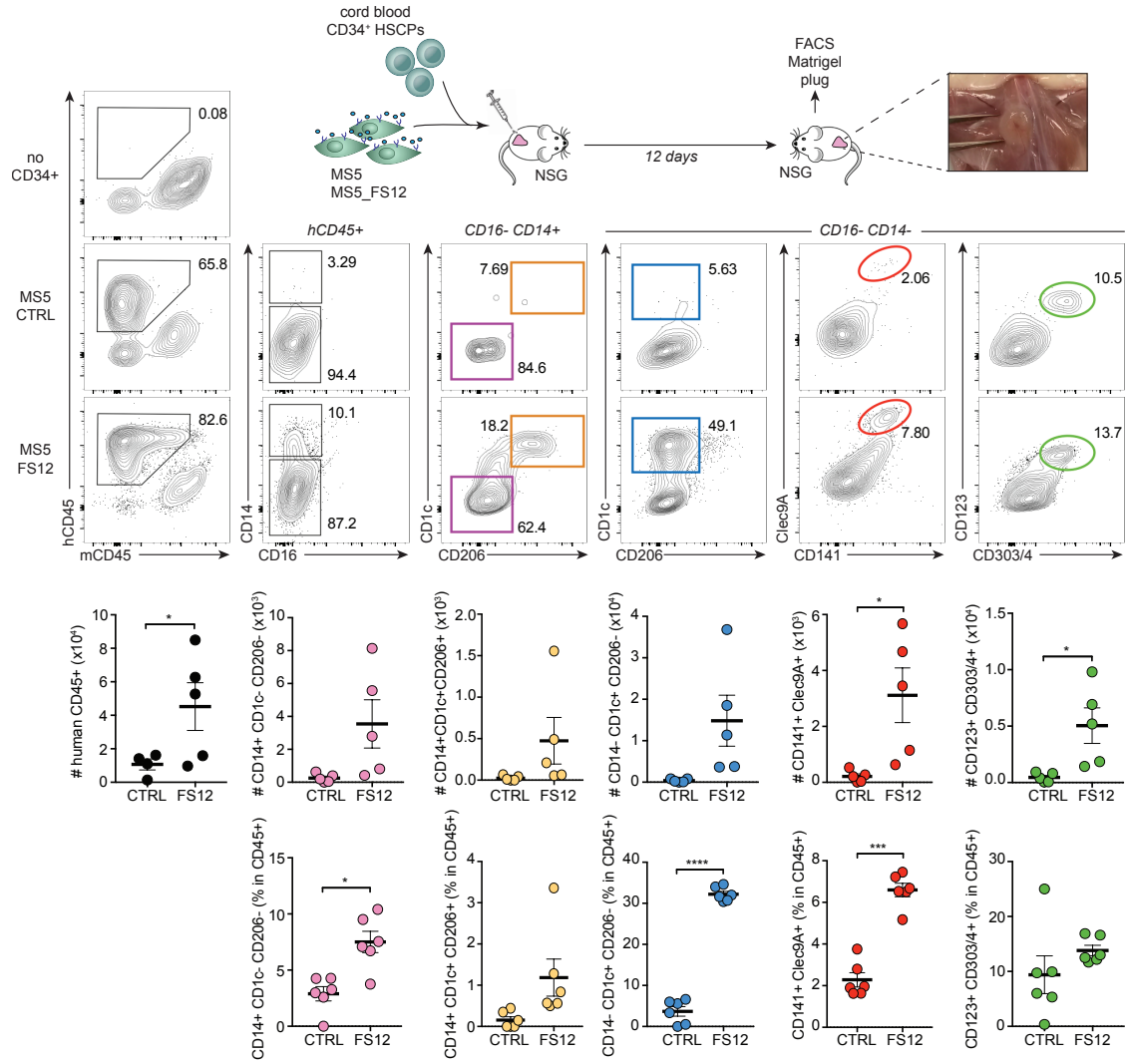


Figure 3-6. Engineered MS5_FS12 promote human DCs differentiation *in vivo* in subcutaneous implants in NSG mice. Flow cytometry plots and quantification of human DCs generated *in vivo* by injecting basement membrane matrix (Matrigel®) preparations containing $CD34^+$ progenitors along with engineered MS5 (MS5_FS12) into NSG mice. Matrigel implants (plugs) were recovered 12 days after injection and the presence of human DCs was assessed by flow cytometry (n=5/6 plugs from 4 mice/group in two independent experiment. Mean ± SEM; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$, paired student's t-test).

3 Discussion

In this chapter, the establishment of a novel approach to promote human DCs differentiation from CD34+ HSPCs is reported.

Based on the knowledge that dendritic cell are constantly replenished by specific progenitors (CDP) that reside in the bone marrow hematopoietic niche, the generation of a "humanised" niche capable of supporting DCs development was hypothesised. To this end, the human homologues of growth factors essential for the maintenance of a functional hematopoietic niche in mouse were identified, consisting in human SCF, CXCL12 and TPO (Table 1-3). Moreover, human Flt3L was also considered due to its fundamental role in DCs development[8,17] as well as the wide expression of its receptor (Flt3) in human hematopoietic progenitors[183,184].

In order to recapitulate the bone marrow niche microenvironment, mouse stromal cell lines (MS5 and OP9, i.e.) were transduced with multiple combinations of retroviral vectors expressing the selected human factors and their ability to support human DCs development was tested *in vitro* using CD34+ cord blood-derived hematopoietic progenitors. Flow cytometry analysis of the generated cells from 3 independent cord blood donors allowed to conclude that the expression of human Flt3L, SCF and CXCL12 in MS5 stromal cells (MS5_FS12) represent the most efficient combination of factors supporting human DCs differentiation *in vitro* (Figure 3-2).

The most commonly used protocol to generated human DCs *in vitro* reported in literature[203] is based on the differentiation of CD34+ hematopoietic progenitors on mitomycin C-treated MS5 supplemented with recombinant human Flt3L, SCF and GM-CSF (recFSG). Therefore a comparison of this approach with the newly generated MS5_FS12 stromal niche was performed. Flow cytometry analysis of the terminally differentiated cells highlighted the superiority of MS5_FS12 in supporting human DCs

differentiation, and this observation was based on the higher yield of DCs obtained (Figure 3-5) as well as on the ability of MS5_FS12 to maintain a pool of CD34+ undifferentiated progenitors until the late stages of differentiation (Figure 3-5c). Moreover, this novel approach clearly demonstrated that GM-CSF, a cytokine extensively used in *in vitro* studies of human DCs[33,198], is dispensable for human DC generation.

Unexpectedly, a consistent and reproducible negative regulation of DCs development by human TPO was observed. Indeed, the addition of TPO in all the cytokines combinations leading to DCs differentiation was able to abrogate the generation of CD141+Clec9A+ and CD123+CD303/4+ cells (Figure 3-2) as well as shifting the balance of CD1c+ cells towards the differentiation of CD14-CD1c+CD206+ cells at the expenses of the CD14-CD1c+CD206- subset (Figure 3-3). These observations were partially in contrast with previously reported protocols based on the use of TPO for human DC subsets differentiation *in vitro*[69,202]. Proietto et al. reported the successful differentiation of human DC subsets from G-CSF-mobilised CD34+ hematopoietic progenitors cultured for 21 days in presence of human recombinant Flt3L (100ng/ml) and TPO (50ng/ml). Flow cytometry analysis provided evidences of the differentiation of CD14-CD11c+CD1b/c+, CD14-CD11c+Clec9A+ and CD14-CD11c-CD123+ cells, described as cDC2, cDC1 and pDC, respectively. Moreover, even though only a limited number of markers was provided for the phenotypic characterisation of these cells, additional experiments assessing the expression of DC-specific transcription factors as well as the production of cytokines in response to activation stimuli and their ability to induce proliferation of CD4+ allogeneic T cells were performed to further support their DC identity[69].

More recently, Balan et al. reported that an initial 7 days expansion of cord blood-derived CD34+ progenitors in medium supplemented with recombinant human Flt3L (100ng/ml), SCF (100ng/ml), IL-3 (20ng/ml) and TPO (50ng/ml) could improve the differentiation of XCR1+ DC1s but failed to give rise to DC2 (pDC not assessed)[202]. However, these protocols significantly differed from the method described here, both in terms of concentration and duration of the TPO stimulation. Therefore, even if it is not possible to rule out a potential positive effect of TPO in the expansion of early DC progenitors, these experiments demonstrated the detrimental effect of TPO in the terminal differentiation of human DC subsets (Figure 3-2).

3.1 Characterisation of human DC subsets generated *in vitro* in stromal cells co-cultures

The phenotypic characterisation of the DC subsets generated using the MS5_FS12 stromal niche supports the conclusion that *in vitro* generated cells closely resemble their *in vivo* blood counterparts. For instance, unlike the ones generated in MS5+recombinant FSG[34,45], CD141+ Clec9A+ cells differentiated in MS5_FS12 do not express CD1c, in agreement with human blood DC1 phenotype (Figure 3-5a). Moreover, the phenotype of CD14- CD1c+ CD206- has been extensively characterised and appeared to be consistent with circulating human DC2 subset (Figure 3-3c and Figure S3-3b).

However, the identification of cell types based on flow cytometry analysis of their phenotype represents a biased approach limited by the restricted number of markers that can be simultaneously assessed. For this reason, future work will aim at perform RNA sequencing (RNAseq) analysis of FACS-sorted subsets generated *in vitro* and compare them to physiologically circulating blood DC subsets. This will allow a more robust validation of the differentiated cells and will enable their comparison with recently

described additional DC subsets[62]. For instance, the presence of a small population of Axl+Siglec6+ cells may be confirmed and further characterised.

On the other hand, functional validation of the cellular output of this *in vitro* system still needs to be completed. Indeed, the responsiveness of CD1c+ cells to TLR4 stimulation has been assessed, but preliminary results suggesting the specific activation of CD14- CD1c+ CD206- and CD14- CD1c+ CD206+ cells by the TLR8 agonist VTX-2337 need to be repeated. If confirmed, this result will further suggest the close relationship of these subsets with circulating blood DC2. Furthermore, the presence of functional and subset-specific TLRs will need to be assessed in the other described subsets, including TLR3 stimulation in CD141+ Clec9A+ DC1 and TLR7 and TLR9 in CD123+ CD303/4+ pDC. More importantly, the detection of specific cytokines secretion, such as IFN-λ for DC1 or IFN-α/β for pDCs, in response to TLR stimulation will further validate the identity of these cells.

3.2 Heterogeneity of *in vitro* generated CD1c+ cells and their GM-CSF dependency

A more refined phenotypic analysis of CD1c+ cells generated *in vitro* using MS5_FS12 stromal cells revealed the presence of at least three different subsets that can be identified based on the expression of CD14 and CD206: CD14- CD1c+ CD206-, CD14- CD1c+ CD206+ and CD14+ CD1c+ CD206+ cells.

Flow cytometry analysis of blood samples from healthy volunteers demonstrated that only the CD14- CD1c+ CD206- cells, a phenotype consistent with *bona fide* DC2, could be detected *in vivo*, whereas both CD14- CD1c+ CD206+ and CD14+ CD1c+ CD206+ cells were absent in healthy donors PBMCs (Figure 3-3b).

In order to characterise the growth factors requirements for their development, the generation of these three subsets was compared with *in vitro* co-cultures of CD34+

progenitors in either MS5_FS12 or MS5 expressing human GM-CSF (MS5_GM) (Figure 3-4a). Flow cytometry analysis of the differentiated cells demonstrated that, unlike CD14- CD1c+ CD206- cells, both CD14- CD1c+ CD206+ and CD14+ CD1c+ CD206+ subsets were successfully generated in the presence of human GM-CSF. More importantly, MS5_GM appeared to be more efficient then MS5_FS12 in inducing the differentiation of CD206+ cells, even if statistical analysis did not reach significance (Figure 3-4b). These results were consistent with preliminary *in vivo* experiments (n=1) in NSG mice, where human PBMCs were injected intravenously in mice bearing B16 melanoma tumors engineered to express the human isoforms of either Flt3L or GM-CSF (Figure S3-4). The analysis of human cells recovered at the tumor site (lung) highlighted the GM-CSF dependency of CD14- CD1c+ CD206+ and CD14+ CD1c+ CD206+ cells. Consistently with *in vitro* data, the differentiation of these two subsets correlated with a reduction in the total number of CD14+ monocytes (Figure 3-4b and Figure S3-4). Moreover, the CD14- CD1c+ CD206- subsets expanded *in vivo* only in response to human Flt3L, as observed *in vit*ro in the MS5_FS12 samples (Figure 3-4b and Figure S3-4).

The heterogeneity of DC2s have been recently described in human[62,96], consistently with previously reported mouse data[21,22]. Yin et al. described the identification of two subsets within the CD1c+ cells displaying different phenotype and function and that can be separated based on the expression of CD5. Gene expression analysis suggested that while $CD5^{hi}$ cells expressed higher levels of DC2-specific genes, $CD5^{low}$ cells preferentially expressed monocyte-related genes[96]. With a different experimental approach Villani et al. also highlighted the existence of two subsets within the CD1c+ cells, defined as DC2 (CD1c+_A) and DC3 (CD1c+_B). DC2 were characterised by the expression of an MHCII-related gene set and described as “non-inflammatory”, whereas

DC3 expressed a CD14+ monocyte-like gene set and was defined as "inflammatory"[62]. Even though this characterisation was mainly based on single-cell RNAseq data in human PBMC, the expression of CD163 was proposed as one of the markers allowing the separation of these two subsets[62]. Therefore, the expression of both CD5 and CD163 was assessed in the CD14- CD1c+ CD206- and CD14- CD1c+ CD206+ cells generated *in vitro* in the MS5_FS12 stromal niche. However, our cells did not align clearly with published subsets (Figure 3-3a Figure S3-3b). Conversely, the phenotype of *in vitro* differentiated CD14+ CD1c+ CD206+ was consistent with the one described for human inflammatory DCs (infDC) by Segura et al. Human infDC were identified in inflammatory fluids such as arthritic synovial fluid or tumor ascites, and based on their transcriptome analysis were proposed to be derived from monocytes[81].

In summary, while CD14- CD1c+ CD206- cells are exclusively dependent on Flt3L *in vitro* and can be expanded from PBMCs *in vivo* in the presence of human Flt3L, the CD14- CD1c+ CD206+ and CD14+ CD1c+ CD206+ cells resulted to be both Flt3L- and GM-CSF-dependent and can be induced *in vitro* and *in v*ivo by human GM-CSF. In addition, the differentiation of both subsets correlates with a reduction of the absolute numbers of CD14+ monocytes *in vitro* and *in vivo* (Figure 3-4 and Figure S3-4). All together these results may suggests a potential monocytic origin of the CD14+ CD1c+ CD206+ and CD14- CD1c+ CD206+ subsets, even though experimental evidences supporting this conclusion are not provided.

In order to decipher the identity of CD14+ CD1c+ CD206+ and CD14- CD1c+ CD206+ cells two main approaches may be considered for future experiments. On one side, RNA sequencing analysis can be performed to characterise the gene expression profiles of these *in vitro* generated subsets and compared them to *ex vivo* isolated inflammatory DCs, for instance from synovial fluid samples of rheumatoid arthritic patients. The

resulting set of data can be also compared to available gene expression profiles for infDC or inflammatory macrophages[60]. Furthermore, a functional characterisation of CD14+ CD1c+ CD206+ and CD14- CD1c+ CD206+ cells can be achieved by assessing their ability to efficiently induce T cells responses. Indeed, it has already been demonstrated that infDC promote T helper 17 (Th17) cells differentiation from naïve CD4+ T cells by secreting Th17 cell-polarising cytokines[60].

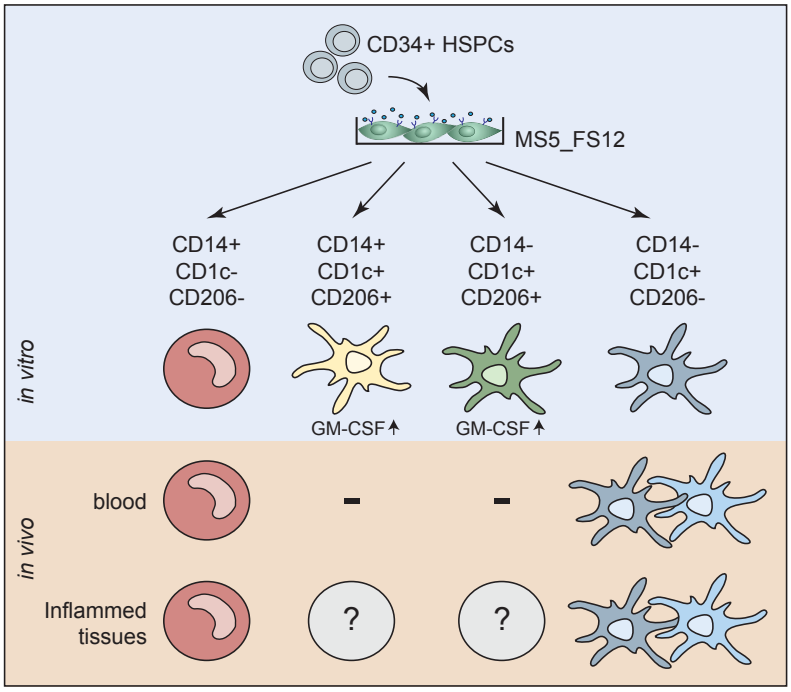


Figure 3-7. Heterogeneity of CD1c+ cells *in vitro* and *in vivo*

3.3 Differentiation of human DCs *in vivo* in NSG mice

Successful generation of human DCs *in vivo* have been achieved by the administration of human recombinant Flt3L in fully reconstituted humanised mice (Table 1-6). However, the mice "humanisation" is a time-consuming process (~ 12 weeks) that requires an initial sub-lethal irradiation of the animals. Therefore the ability of the MS5_FS12 stromal niche to support human DCs differentiation *in vivo* was tested. Flow cytometry analysis of the cells recovered from subcutaneous Matrigel-based implants

containing human CD34+ progenitor along with MS5_FS12 cells demonstrated the generation of CD141+Clec9A+, CD14-CD1c+ and CD123+CD303/4+ cells as early as day 12 in NSG mice (Figure 3-6).

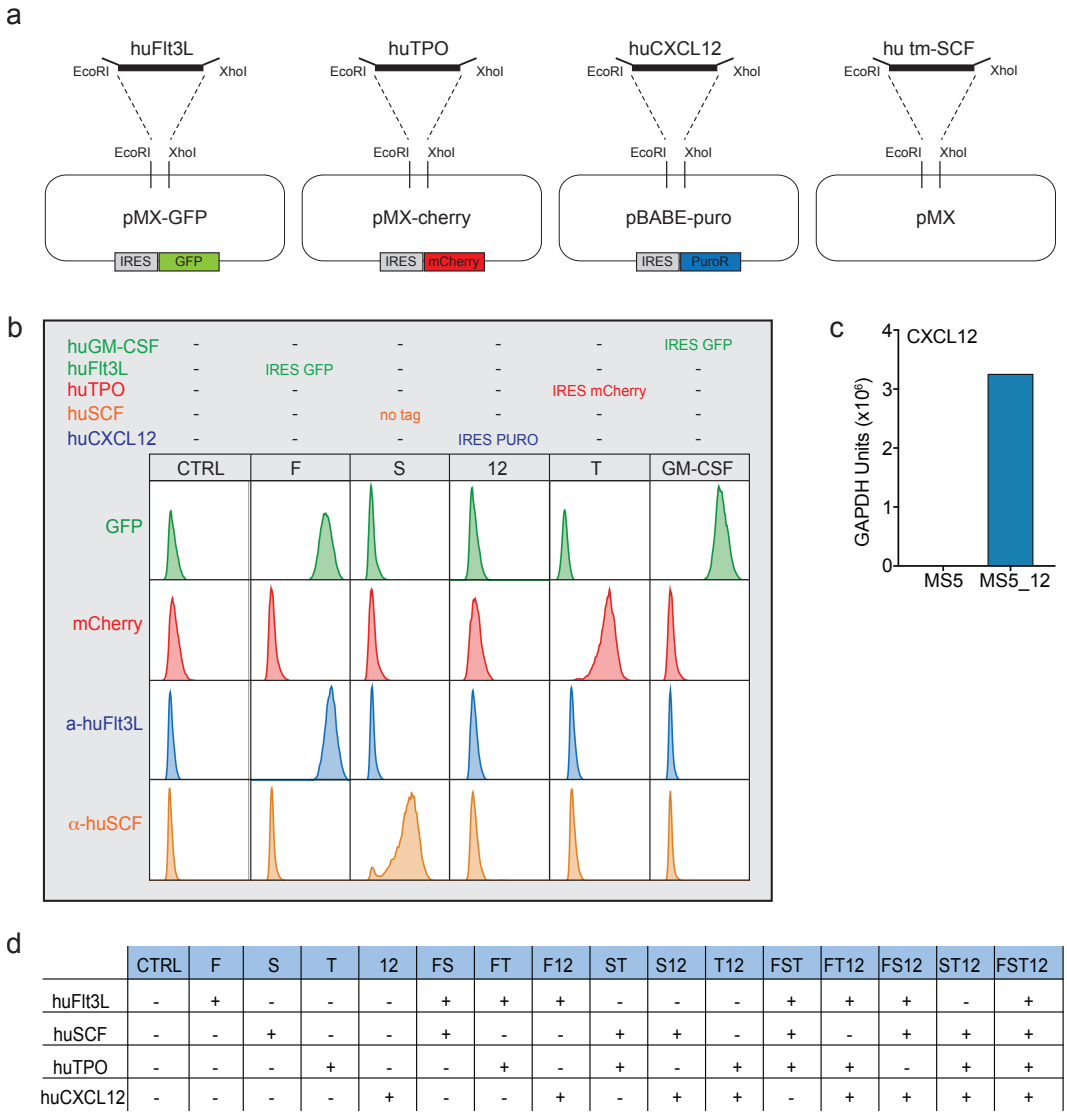
However, in order to functionally validate the *in vivo* differentiated cells, future experiments will aim at testing their ability to efficiently activate human T cell. Such approach will also provide a platform to study human DCs interaction with T cells *in vivo*. Therefore, preliminary experiments were performed to test whether intravenously injected human T cells were capable of migrate towards the subcutaneous Matrigel-based implant and interact with the *in vivo* differentiated human DCs. As shown in Figure S3-6, cell trace violet (CTV)-labelled T cells were injected intravenously in NSG mice bearing a subcutaneous implant (plug) containing human DCs (day 12). Flow cytometry analysis 4 days after T cells injection revealed the presence of human CD45+ cells in both spleen and Matrigel plug. However, non-dividing CTV+CD3+ T cells were only detected in the spleen of the animals, demonstrating their inability to reach the plug and interact with human DCs (Figure S3-6).

An alternative approach to assess the function of *in vivo* differentiated human DCs will be used in future experiments, based on the assessment of antigen-specific T cells proliferation *in vivo*. To this end, human T cells will be transduced with a retroviral vector (pMSGV1-F5AfT2aB) coding for the a and b chain of the F5 anti-MART1/A2 TCR[670]. Subsequently, CD34+ HSPCs from an HLA-A02*01+ cord blood donor will be differentiated *in vivo* using MS5_FS12 stromal cells in subcutaneous Matrigel plugs. After 12 days of differentiation, UV-killed cell lines expressing the melanoma antigen MART1 will be injected into the plug as a source of antigen for the *in vivo* differentiated DCs. This step will be followed by the injection of MART1 transgenic T

cells and the antigen-specific proliferation will be measured by CTV dilution of the T cells recovered from the plug.

This system will provide a functional validation of the generated human DCs by testing their ability to cross-present exogenous antigens. Moreover, it may represent a very useful platform enabling the study of human T cells interaction with DCs *in vivo*, for instance in the context of anti-tumor immune responses.

4 Supplementary figures



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	CTRL	F	S	T	12	FS	FT	F12	ST	S12	T12	FST	FT12	FS12	ST12	FST12
huFlt3L	-	+	-	-	-	+	+	+	-	-	-	+	+	+	-	+
huSCF	-	-	+	-	-	+	-	-	+	+	-	+	-	+	+	+
huTPO	-	-	-	+	-	-	+	-	+	-	+	+	+	-	+	+
huCXCL12	-	-	-	-	+	-	-	+	-	+	+	-	+	+	+	+

Figure S3-1. Generation of engineered stromal cell lines (MS5) expressing human cytokines. (a) Strategy to clone human bone marrow niche factors into retroviral vectors (pMX). **(b)** Validation of FACS-sorted lines expressing single cytokines by flow cytometry, based on the expression of fluorescent reporters and antibody staining of trans-membrane proteins (huFlt3L and huSCF, i.e.). **(c)** Validation of human CXCL12 expression by qPCR in MS5_12 after puromycin selection. **(d)** Engineered MS5 lines generated by combinatorial expression of human cytokines Flt3L (F), SCF (S), TPO (T), CXCL12 (12) and GM-CSF.

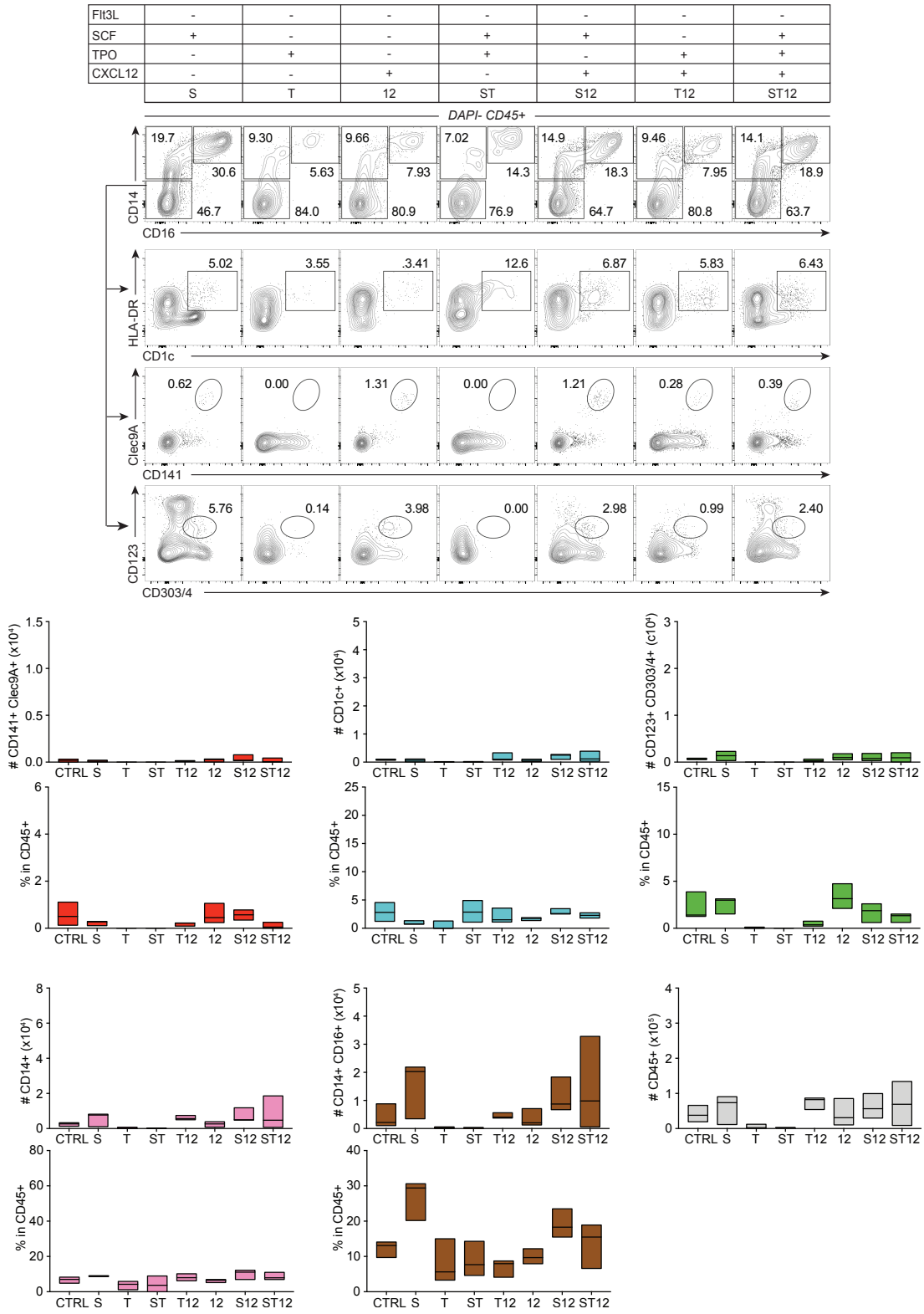


Figure S3-2. Human DC differentiation strictly depends on Flt3L and it is improved by the presence of the niche factors SCF and CXCL12. Representative flow cytometry plots of $CD34^+$ progenitors differentiated *in vitro* on engineered MS5 lines for 15 days in absence of huFlt3L. Quantification of *in vitro* generated cells expressed as absolute number and percentages of human $CD45^+$ cells (n=3 independent cord blood donors. Mean ± SEM; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, one-way ANOVA test).

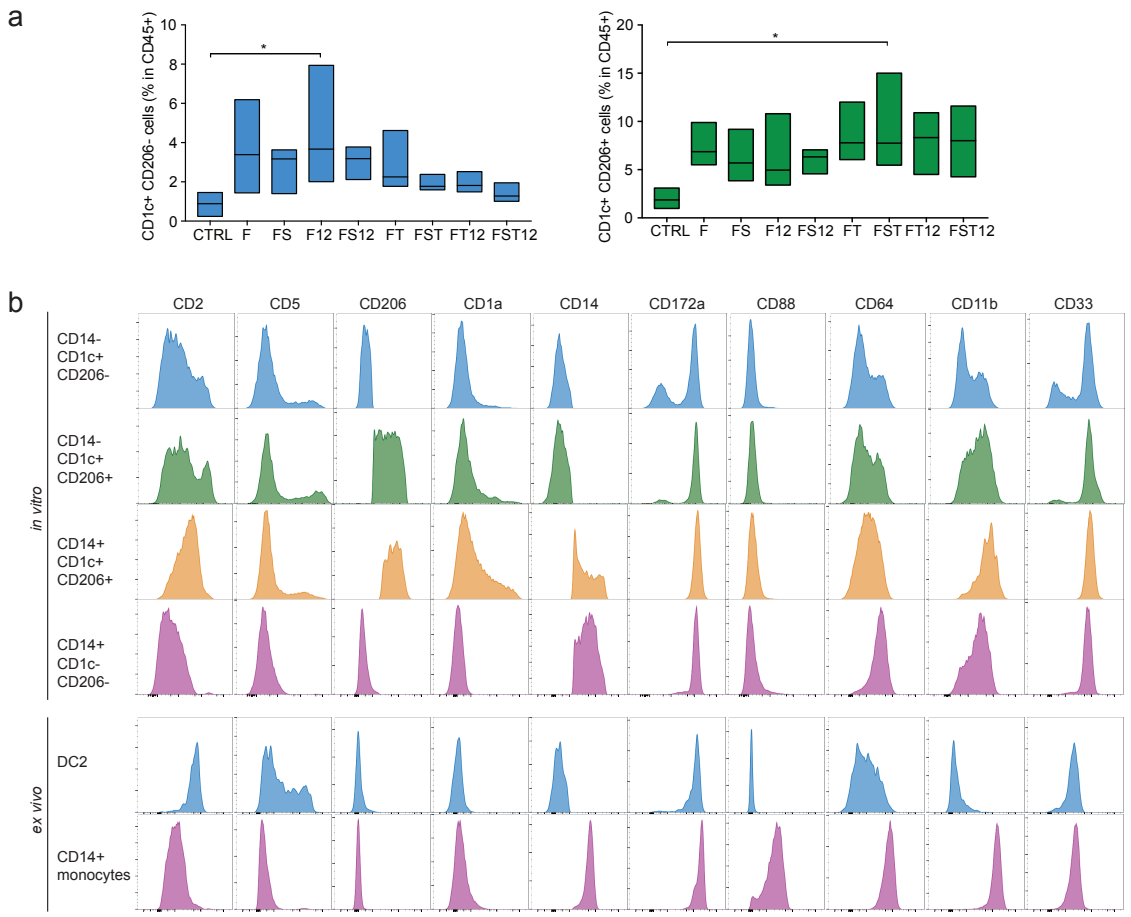


Figure S3-3. *In vitro* differentiated CD1c$^+$ cells are heterogeneous. (a) Quantification of *in vitro* generated CD1c$^+$ cells from CD34$^+$ HSPCs co-cultured with huFlt3L-producing MS5 lines expressed as percentages of human CD45$^+$ cells (n=3 independent cord blood donors. Mean ± SEM; * $p<0.05$, one-way ANOVA test). **(b)** Histograms of a representative experiment showing cell surface markers expressed in human monocytes and CD1c$^+$ cells generated *in vitro* with MS5_FS12 as compare to cells isolated from blood.

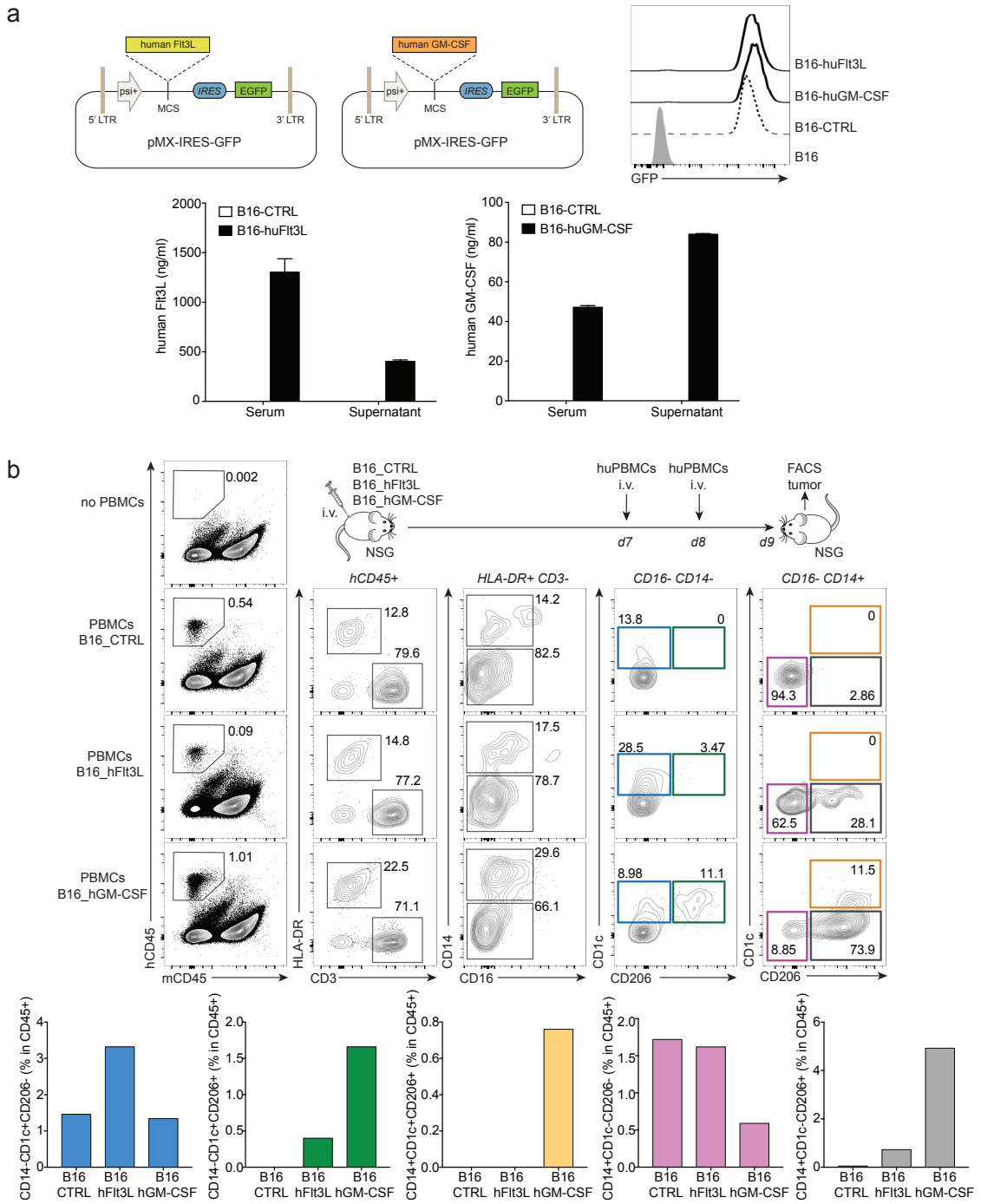


Figure S3-4. MS5_FS12 support the differentiation of CD1c^{+} CD206^{-} cells that cannot be generated with GM-CSF alone *in vitro* and *in vivo*. (a) Generation of mouse melanoma B16 cells expressing human Flt3L and human GM-CSF by retroviral transduction (pMX-IRES-GFP vector) and validation by assessing cytokines secretion *in vitro* (supernatant) and *in vivo* (serum) by ELISA. **(b)** Intravenous (i.v.) injection of mouse melanoma cells (B16) expressing human Flt3L and GM-CSF into NSG mice followed by i.v. injection of human PBMCs at days 7 and 8. Metastatic tumors in the lungs were collected at day 9 and the presence of human cells was evaluated by flow cytometry (n=1 mouse per condition).

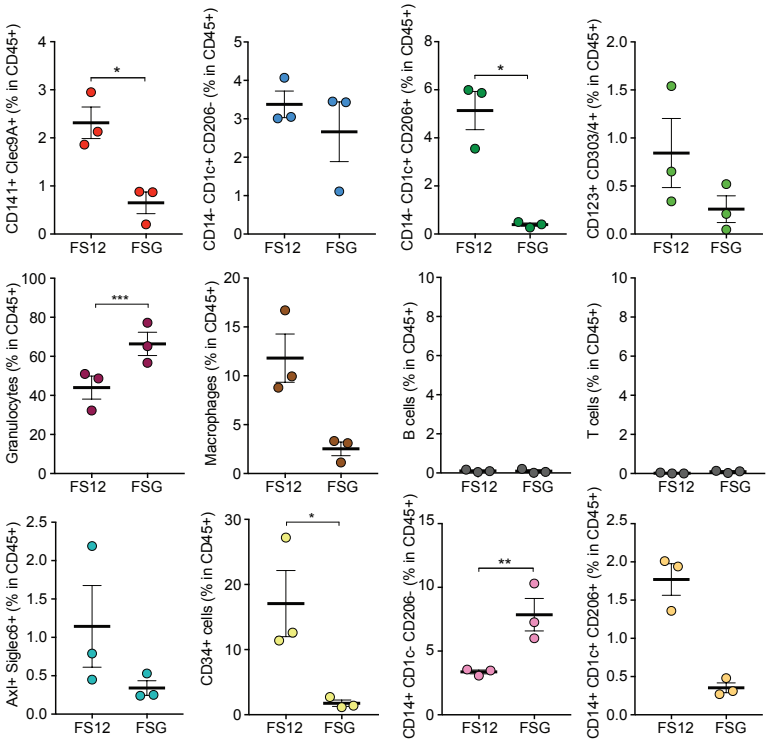


Figure S3-5. MS5_FS12 are superior to recombinant FSG to support human DCs differentiation from cord blood-derived hematopoietic progenitors. Quantification of *in vitro* generated cells from $CD34^+$ HSPCs co-cultured with MS5 expressing human Flt3L, SCF and CXCL12 (FS12), as compared to mitomycin-treated MS5 supplemented with recombinant Flt3L, SCF and GM-CSF (FSG) (n=3 independent cord blood donors. Mean ± SEM; * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$ two-way ANOVA test).

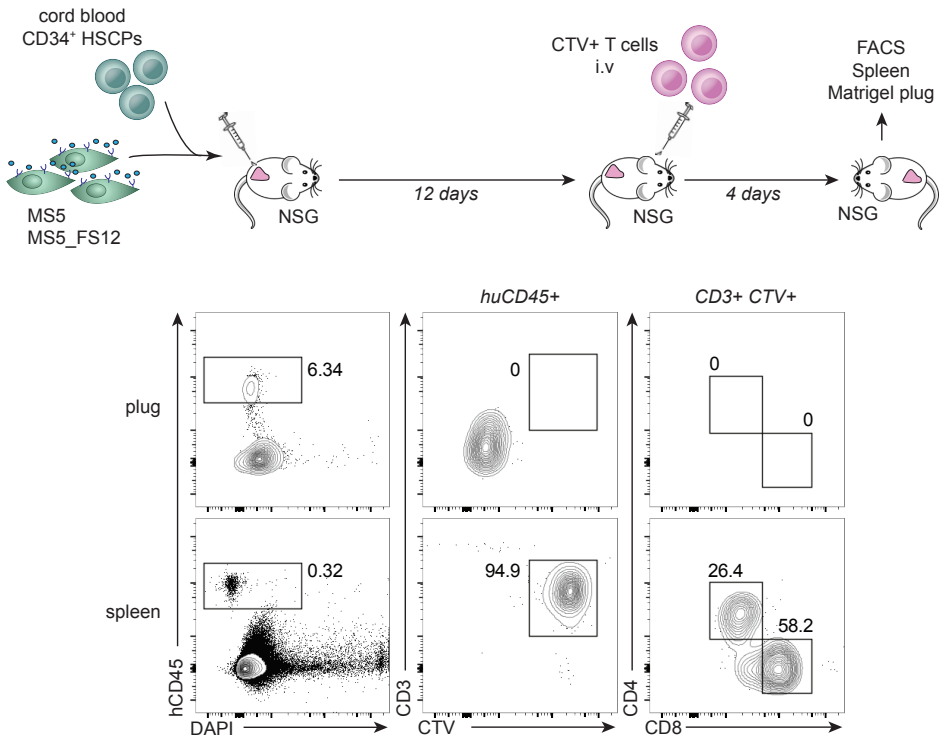


Figure S3-6. Engineered MS5_FS12 promote human DCs differentiation *in vivo* in subcutaneous implants in NSG mice. Human T lymphocytes were injected intravenously in NSG mice carrying subcutaneous Matrigel implants containing human DCs differentiated *in vivo* from cord-blood-derived CD34+ progenitors using MS5_FS12 stromal cells. Four day after T cells injection, the presence and proliferation of human T cells in spleen and Matrigel plugs was assessed by flow cyometry.

Chapter 4 Modelling human definitive hematopoiesis using iPSC

1 Introduction and objectives

The establishment of a reliable system to differentiate human DC subsets from iPSCs represents an invaluable platform to study human DC biology. Indeed, the possibility to generate human DCs from an inexhaustible source of genetically matched or patient-specific cell lines in combination with highly efficient genome editing techniques such as CRISPR/Cas9, would enable a better understanding of human DCs biology and their potential application in clinical therapies. In the last decade, numerous studies reported the generation of DCs from human PSCs (either ESC or iPSC)[656,659,660,664–666]. However, a full characterisation of their phenotype, function and hematopoietic origin was not provided.

DCs originate from HSCs and they are constantly replenished by bone marrow precursors (CDP) during adult life. Therefore, the induction of a definitive hematopoietic program during differentiation of pluripotent stem cells is essential to drive the development of human DCs. Numerous publications described the differentiation of hematopoietic progenitors from pluripotent stem cells mainly based on two approaches: (i) the formation of embryoid bodies (EB) in the presence of factors driving hematopoietic differentiation; (ii) the co-culture of PSC with mouse bone marrow stromal cells supporting hematopoietic differentiation (OP9 or MS5, e.g.). Both systems have been quite extensively characterised, and the generation of a variety of terminally differentiated cells have been reported. However, no reliable marker has been identified to successfully discriminate between primitive and definitive progenitors at early stages of differentiation, and the assessment of the potential of those cells still represents the best validation of their primitive or definitive origin.

The goal of this chapter is to develop an *in vitro* system mimicking the embryonic events leading to the generation of definitive hematopoietic progenitors, from the initial mesoderm commitment through the differentiation of hemangioblast-like progenitors and resulting in the formation of hemogenic endothelial cells, which undergo endothelial-to-hematopoietic transition (EHT) and give rise to hematopoietic progenitors. Promoting the definitive hematopoietic program while recapitulating these early embryonic events *in vitro* may represent a successful strategy to induce human DCs differentiation.

1.1 Objectives

In order to establish an *in vitro* protocol to generate human iPSC-derived DC subsets the objectives of this chapter were:

- To develop an *in vitro* culture system to promote the differentiation of definitive hematopoietic stem and progenitors cells from human iPSC;
- To assess the potential of *in vitro* differentiated hematopoietic progenitors to generate human DC subsets *in vitro* and *in vivo*;

2 Results

2.1 OP9 stromal cells expressing human Flt3L induce SOX17 up-regulation in iPSC-derived cells *in vitro*

Mouse stromal cells have been extensively used to support hematopoietic differentiation of human PSCs. In order to test if human growth factors involved in hematopoietic maintenance and DCs development *in vitro* may play a role in the early stages of embryonic haematopoiesis, mouse stromal cells constitutively expressing the human Flt3L, SCF and CXCL12 were generated. For this reason, a first comparison of two

mouse bone marrow-derived stromal cell lines, MS5 and OP9, has been performed to evaluate their ability to induce the differentiation of CD34+ cells from iPSC. Flow cytometry analysis at day 10, 12 and 14 of differentiation using two iPSC lines (iKCL4 and iKCL11) demonstrated that OP9 cells are superior to MS5 in generating CD34+ cells, as shown by the statistically significant increased of the total number of cells generated (Figure 4-1a). Moreover, due to the higher yield of CD34+ cells, day 12 was chosen as the best time-point for all future experiments. Based on these results, OP9 cells were transduced with retroviral vectors expressing human Flt3L (pMX-IRES-mCherry), SCF (pMX) and CXCL12 (pBABE-Puro). After transduction, cells were FACS-sorted and validated based on the expression of fluorescent reporters as well as antibody staining of trans-membrane proteins (SCF, i.e.) (Figure 4-1b). The potential of these lines to support the differentiation of CD34+ cells was first assessed using two iPSC lines (iKCL4 and iKCL11) and no significant difference was observed in the total number and frequency of live cells as compared to regular OP9 (OP9_CTRL) (Figure 4-1c). However, to evaluate if any variation in the transcriptional profile of these cells was induced by the presence of human growth factors, the expression of genes involved in different stages of hematopoietic commitment during embryonic development was assessed by qPCR. Messenger RNA extracted from bulk populations of cells obtained from iPSC co-cultures with engineered OP9 (OP9_F, OP9_S and OP9_12) was used. A first validation of the ability to detect human cDNA as well as the specificity of the qPCR primers was performed in iPSC samples differentiated on OP9 and cord blood-derived CD34+ progenitors, respectively (Figure S4-1a-b-c). Subsequently, the expression of genes known to be up-regulated in hemogenic endothelium (SOX17), definitive hematopoietic cells (RUNX1, MYB) and early and late hematopoietic precursors (PU1, GATA1, GATA2, Id2, IRF8) was evaluated by qPCR (Figure 4-1d

and Figure S4-1d). Gene expression results highlighted the significant up-regulation of SOX17 expression in samples differentiated in the presence of human Flt3L (Figure 4-1d). Moreover, even if hematopoietic genes were induced in all the engineered OP9 samples when compared to undifferentiated iPSCs, no significant difference was observed among the tested cytokines (Figure 4-1d and Figure S4-1d).

In conclusion, even though no difference was observed in the yield of CD34+ cells generated from engineered OP9 (OP9_F, OP9_S and OP9_12) when compared to OP9_CTRL, a statistically significant up-regulation of the hemogenic endothelium transcription factor SOX17 was detected in OP9_F. The same condition also induced a consistent but not significant increased in the expression of the myeloid transcription factor PU1.

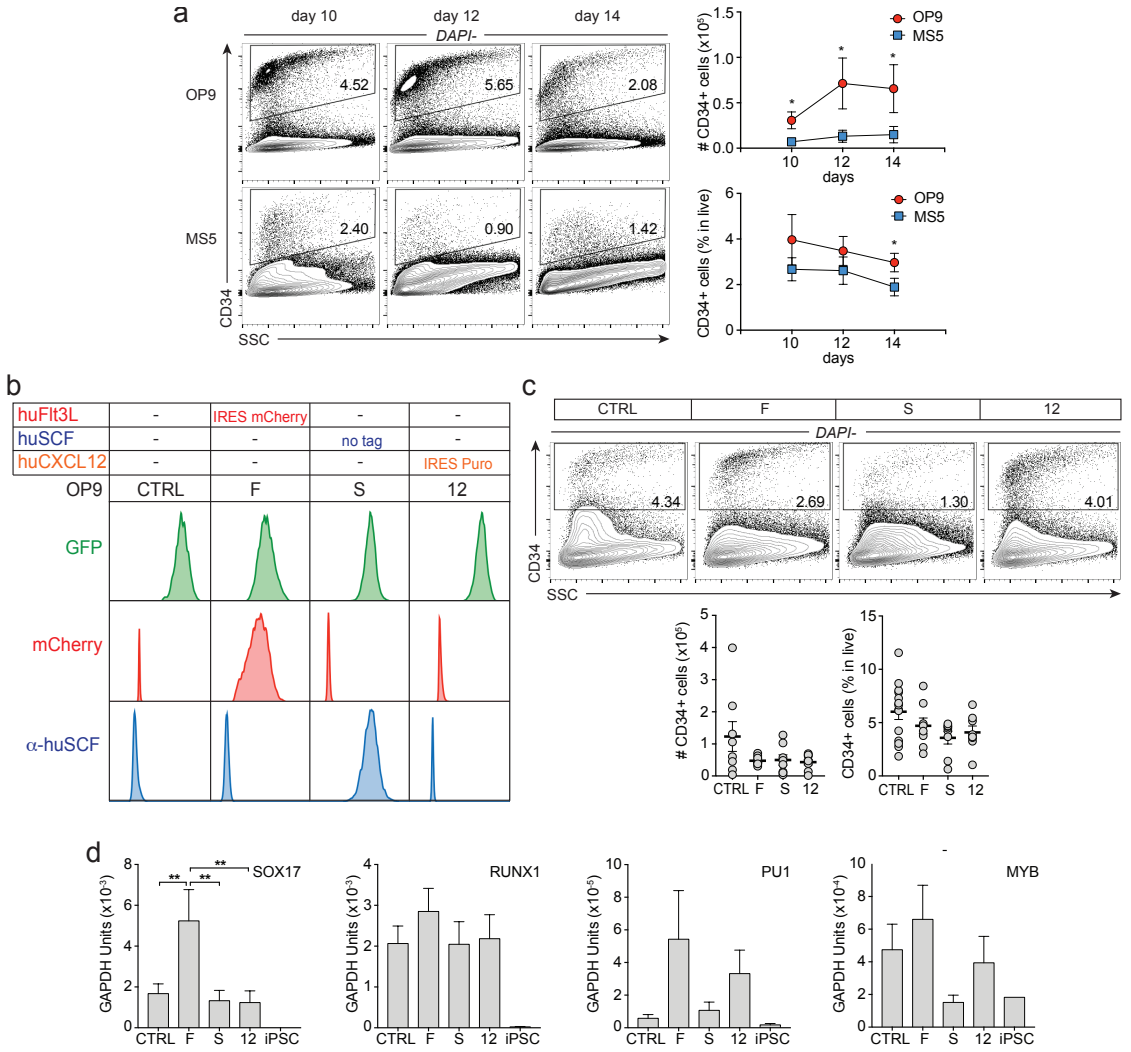


Figure 4-1. OP9 stromal cells expressing human Flt3L induce SOX17 expression in cells differentiated from human iPSC. (a) Hematopoietic differentiation of human iPSCs using mouse stromal cell lines OP9 and MS5 in a time-course experiment. The generation of CD34+ cells from two human iPSC lines (iKCL4 and iKCL11) was assessed by flow cytometry (n=8-10 technical replicates from 7 independent experiments for each time-point. Mean ± SEM; * $p<0.05$, paired student's t-test). **(b)** Validation of FACS-sorted engineered OP9 stromal cells expressing human Flt3L (F), SCF (S) and CXCL12 (12) by flow cytometry. **(c)** Differentiation of human iPSC into CD34+ cells using OP9 stromal cells (CTRL) and engineered OP9 expressing human Flt3L (F), SCF (S) and CXCL12 (12) at day 12. Quantification of n=9 technical replicates from 6 independent experiments using two iPSC lines (iKCL4 and iKCL11) expressed as absolute number of cells and frequency of live cells (Mean ± SEM; * $p<0.05$, **$p<0.01$, Kruskal-Wallis test). **(d)** Gene expression analysis by qPCR of cDNA samples obtained by reverse transcription of mRNA extracted from bulk cell populations resulting from 12 days of iPSC co-culture with engineered OP9 stromal cells (n=7 technical replicates from 6 independent experiments using two iKCL4 and iKCL11 iPSC lines. Mean ± SEM; ** $p<0.01$, one-way ANOVA test).

2.2 Human Flt3L exclusively induces the differentiation of CD43+CD41+CD90+ hematopoietic cells from iPSC

In order to further understand the consequences of Flt3L expression in the differentiation of hematopoietic progenitors from iPSC, a more extensive phenotypic analysis of the generated progenitors was performed by flow cytometry. To this end, the expression of surface proteins marking either endothelial or hematopoietic commitment was evaluated in iPSC-derived CD34+ cells generated in OP9_CTRL, OP9_F, OP9_S and OP9_12 stromal lines. The expression of CD73 was first evaluated to discriminate between endothelial (CD73+) and non-endothelial progenitors (CD73-). Within the CD73- subset, the combination of CD43 and CD90 expression was used to identify cells committed to the hematopoietic fate (CD43+) and cells displaying a hemogenic endothelium phenotype (CD34+CD73-CD43-CD90+). Finally, the expression of CD41 and CD90 was evaluated within the CD43+ compartment, in order to detect the presence of early hematopoietic progenitors (gating strategy in Figure 4-2a). No significant difference was observed in the absolute number and frequency of the CD43+ cells as well as the CD73-CD43-CD90+ HE cells among the different engineered stromal lines tested (Figure 4-2b and Figure S4-2a). Conversely, a significant increase in the frequency of CD43+CD41+ cells was exclusively observed in cells differentiated in OP9_F stromal cells, and this was consistent with a significant reduction of the CD43+CD41- subset (Figure 4-2b and Figure S4-2a). A more in-depth analysis of the CD43+CD41+ population highlighted the existence of a CD43+CD41+CD90+ subset, whose absolute numbers and frequency within CD34+ and CD73- cells was significantly increased in OP9_F (Figure 4-2b).

All together these results demonstrated the existence of a CD43+CD41+CD90+ subset of cells, which is exclusively induced by the expression of human Flt3L by OP9 stromal cells.

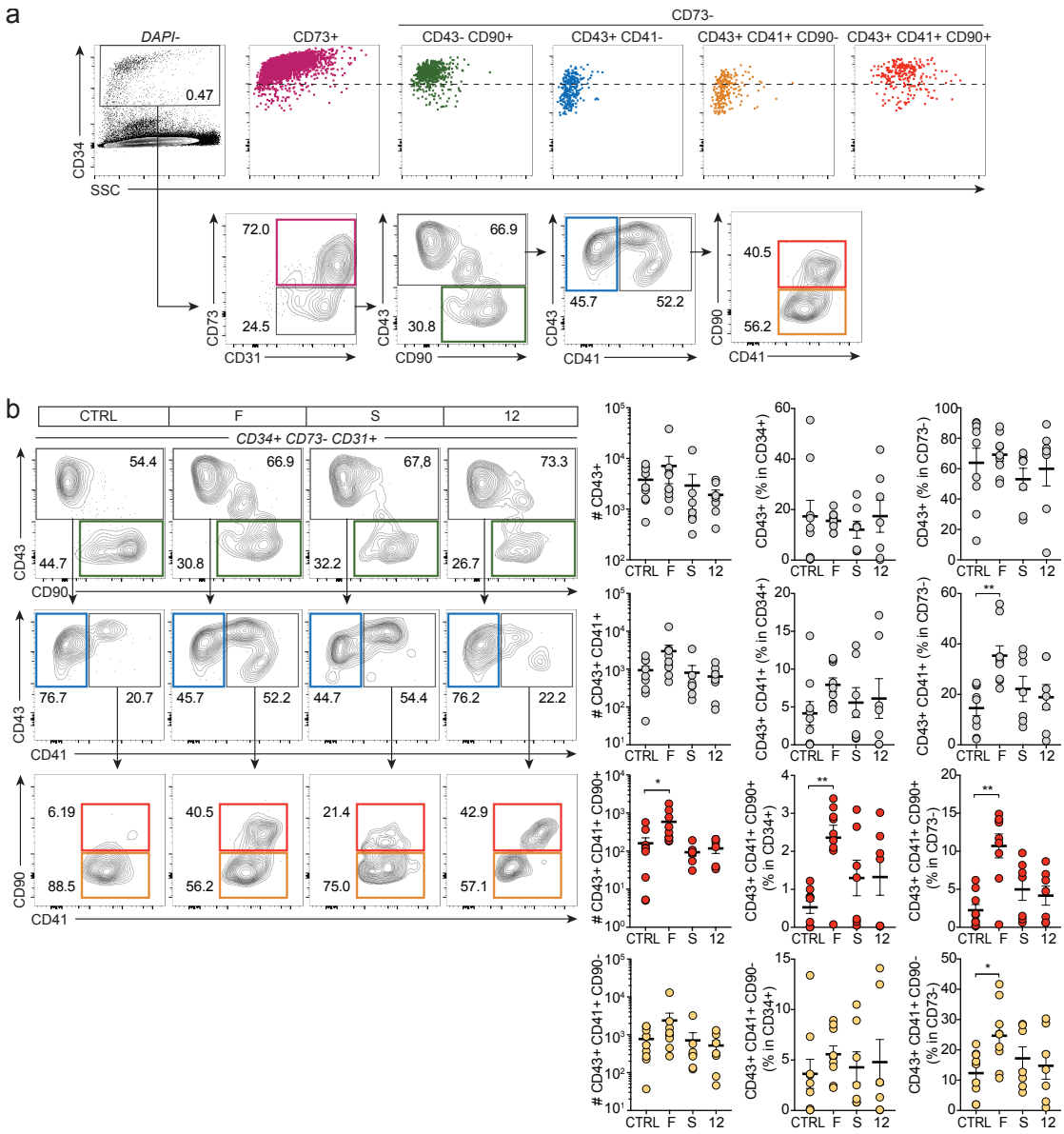


Figure 4-2. OP9 stromal cells expressing human Flt3L exclusively induce the differentiation of CD43+ CD41+ CD90+ hematopoietic cells from human iPSCs. (a) Gating strategy to identify iPSC-derived endothelial and hematopoietic cells and representative example of CD34+ expression levels in each subpopulation. **(b)** Flow cytometry analysis of endothelial and hematopoietic subpopulations generated by culturing human iPSC for 12 days in the presence of OP9 (CTRL) and OP9 expressing human Flt3L (F), SCF (S) and CXCL12 (12). Quantification of absolute number of cells, frequency of CD34+ cells and frequency of CD73- non-endothelial cells for n=7-9 technical replicates from 4 independent experiments using iKCL4 and iKCL11 lines (Mean ± SEM; * $p<0.05$, ** $p<0.01$, one-way ANOVA test).

2.3 Human Flt3L and SCF promote the differentiation of arterial vascular endothelium from human iPSCs

The development of both endothelial and hematopoietic progenitors during embryogenesis are strictly correlated. Therefore, phenotypic analysis of the CD73+ endothelial-committed progenitors generated *in vitro* using OP9, OP9_F, OP9_S and OP9_12 was performed by flow cytometry. To this end, the expression of CD184 (CXCR4) was evaluated (gating strategy in Figure 4-3a), as endothelial progenitors' potential to differentiate in arterial (CD184+) or venous (CD184-) vascular endothelium correlates with the expression of CD184. No significant variation was observed in the absolute number and frequency of CD73+ cells among the different engineered OP9 lines tested. However, qualitative analysis of these cells highlighted a significant increase of the CD184+ fraction in both OP9_F and OP9_S, corresponding to a reduction of CD184- subset frequency within CD34+ and CD73+ cells (Figure 4-3b-c). These results correlated with a consistent but not significant increase of the expression of CXCR4 mRNA as measured by qPCR (Figure 4-3d).

Hence, the stromal cells-derived human Flt3L and SCF were capable to influence the development of iPSC-derived endothelial progenitors, by promoting the differentiation of arterial vascular endothelium at the expenses of the venous counterpart.

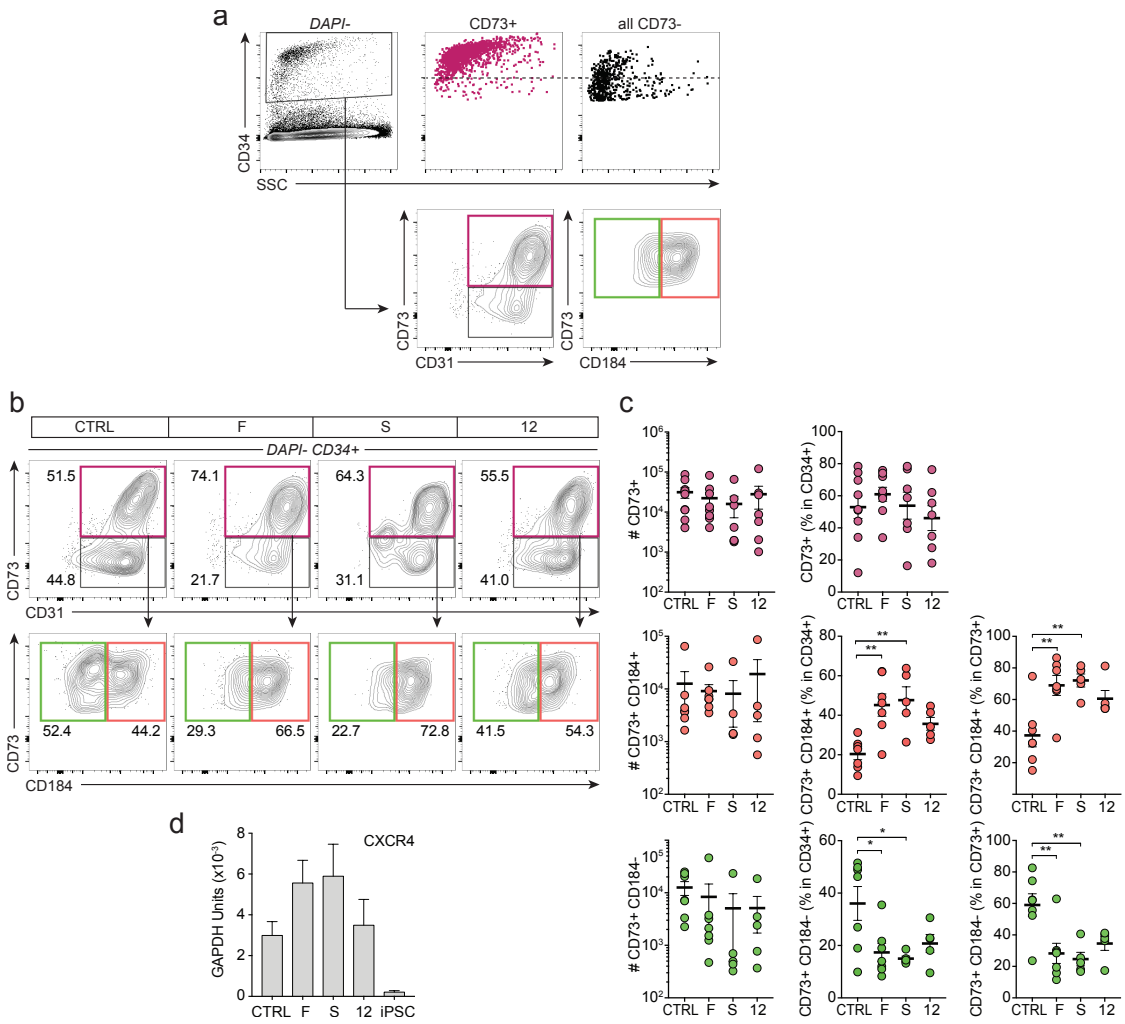


Figure 4-3. OP9 stromal cells expressing human Flt3L and SCF promote arterial vasculature differentiation from iPSCs. (a) Gating strategy to assess heterogeneity of iPSC-derived endothelial cells and representative example of CD34+ expression levels in each subpopulation. **(b)** Flow cytometry analysis of CD34+ CD31+ CD73+ endothelial cells generated in iPSC co-cultures with OP9 (CTRL) and OP9 expressing human Flt3L (F), SCF (S) and CXCL12 (12) at day 12 of differentiation. **(c)** Quantification of absolute number of cells, frequency of CD34+ cells and frequency of CD73+ endothelial cells for n=7-9 technical replicates from 4 independent experiments using iKCL4 and iKCL11 lines (Mean ± SEM; * $p<0.05$, ** $p<0.01$, one-way ANOVA test). **(d)** Quantitative PCR analysis of CXCR4 (CD184) expression in iPSC-derived cells at day 12 of differentiation (n=7 technical replicates from 6 independent experiments using two iKCL4 and iKCL11 iPSC lines)**.**

2.4 Human Flt3L promotes the differentiation of iPSC-derived CD34+ progenitors capable of generating CD14-CD1c+ cells *in vitro*

In order to test whether the presence of human Flt3L produced by OP9 stromal cells would affect the potential of iPSC-derived progenitors to differentiate into human DC subsets, CD34+ cells differentiated using OP9_CTRL and OP9_F were sorted by magnetic beads separation and re-plated on either MS5 (MS5_CTRL) or MS5 expressing human Flt3L, SCF, TPO and CXCL12 (MS5_FST12). Terminally differentiated cells were analysed by flow cytometry at day 15, assessing the presence of human CD45+ hematopoietic cells as well as the expression of markers characterising human DC subsets. Human CD45+ hematopoietic cells were detected in all conditions, even though higher numbers were generated in MS5_FST12 samples. Likewise, all samples supported the differentiation of CD14+ cells, which were increased when human cytokines were present (MS5_FST12). On the contrary, only iPSC-derived CD34+ progenitors generated in OP9_F stromal cells were capable to give rise to CD14-CD1c+ cells, and this was improved when the terminal differentiation was performed using MS5_FST12 (Figure 4-4a-b).

In conclusion, based on one preliminary *in vitro* experiment, the presence of human Flt3L in the early differentiation of iPSC into CD34+ progenitors correlated with the ability of these cells to generate CD14-CD1c+ cells when cultured on MS5 and MS5_FST12. (Figure 3-2).

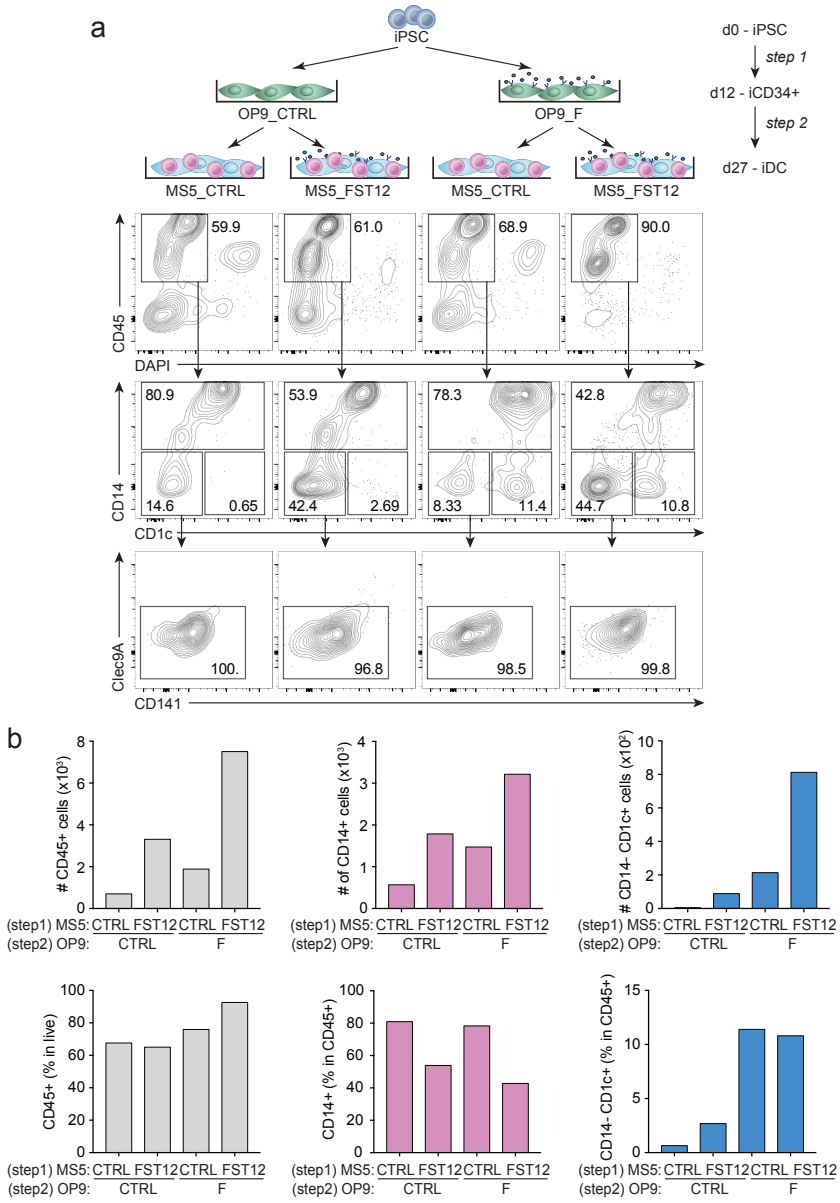


Figure 4-4. OP9 stromal cells expressing human Flt3L promote the differentiation of iPSC-derived CD34+ hematopoietic progenitors capable to generate CD14- CD1c+ cells *in vitro*. (a) Experimental strategy and flow cytometry panels of *in vitro* generated iPSC-derived CD14+ and CD14+ CD1c+ cells. Human iPSC were first differentiated into CD34+ progenitors using OP9 (OP9_CTRL) and OP9 expressing human Flt3L (OP9_F). At day 12 of differentiation, CD34+ cells were isolated by magnetic beads separation and further differentiated *in vitro* using either MS5 (MS5_CTRL) or MS5 expressing human Flt3L, SCF, TPO and CXCL12 (MS5_FST12) for 15 days. **(b)** Quantification of cellular output expressed as absolute number of cells and frequency of CD45+ cells (n=1).

2.5 Human Flt3L promotes the differentiation of iPSC-derived CD34+ progenitors displaying *in vivo* hematopoietic potential in NSG mice

To test the *in vivo* potential of iPSC-derived hematopoietic progenitors differentiated using OP9, OP9_F, OP9_S and OP9_12, CD34+ cells generated in each OP9 stromal line were isolated by magnetic separation and injected subcutaneously in NSG mice along with Matrigel and MS5_FS12 stromal cells. Matrigel implants were recovered 12 days after injection and flow cytometry analysis was performed to detect the presence of differentiated human CD45+ hematopoietic cells.

Among all the tested samples, only CD34+ progenitors generated using OP9_F stromal cells were capable of differentiate in CD45+ hematopoietic cells (Figure 4-5). Further analysis of human CD45+ cells showed that 52% of these were expressing the monocyte-macrophage markers CD14 and CD16, whereas within the CD14-CD16- population approximately half of the cells were CD1c+ (62.8%) while only a fraction (30.1%) appeared to be positive for CD141 expression (Figure 4-5).

Overall this preliminary experiment (n=1) suggested that hematopoietic progenitors generated from iPSC in the presence of human Flt3L (OP9_F) exclusively included a population of cells capable of giving rise to terminally differentiated CD45+ hematopoietic cells *in vivo*.

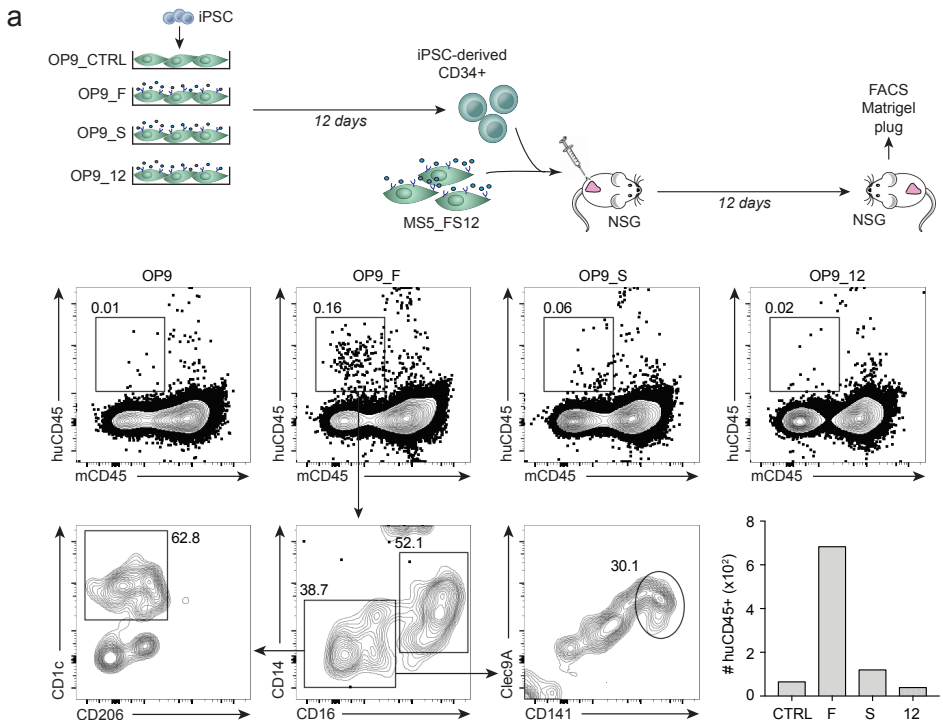


Figure 4-5. OP9 stromal cells expressing human Flt3L generate iPSC-derived CD34+ progenitors displaying *in vivo* hematopoietic potential in NSG mice. (a) *In vivo* differentiation of iPSC-derived CD34+ cells from OP9 and engineered OP9 co-cultures. Human iPSC were differentiated into CD34+ cells on OP9 (CTRL) and OP9 expressing human Flt3L (F), SCF (S) and CXCL12 (12). At day 12 of differentiation, CD34+ cells were isolated by magnetic beads separation and injected subcutaneously into NSG mice along with MS5 (MS5_CTRL) or MS5 expressing human Flt3L, SCF and CXCL12 (MS5_FS12) in basement membrane matrix (Matrigel®) preparations. Cells were recovered from Matrigel implants after 12 days and flow cytometry analysis of human hematopoietic cells was performed (n=1 mouse/group).

3 Discussion

The generation of iPSC-derived dendritic cells requires the induction of a definitive hematopoietic program. Therefore, the ability of an engineered feeder-based approach to promote the development of definitive progenitors capable of DCs differentiation was tested. Mouse bone marrow stromal cells (OP9) expressing human hematopoietic growth factors Flt3L, SCF and CXCL12 were generated by retroviral transduction, and their potential to support hematopoietic differentiation was assessed in co-culture experiments with human iPSC.

Flow cytometry analysis of the resulting iPSC-derived CD34+ cells highlighted an essential role of human Flt3L in the development of a subset of CD34+ CD73- CD43+ CD41+ CD90+ cells, a phenotype consistent with multipotent progenitors characterised by high HSC activity both in human cord blood and iPSC-derived hematopoietic cells[623,671,672]. This effect appeared to be specific for human Flt3L, and was not observed when human SCF or CXCL12 were used (Figure 4-2). Moreover, human Flt3L also promoted the differentiation of arterial vascular endothelium, as demonstrated by the significant higher frequency of CD73+ CD184+ cells corresponding to a reduction of the CD73+ CD184- (venous) fraction of iPSC-derived endothelial progenitors (Figure 4-3). A similar effect was also described when human SCF was provided (Figure 4-3).

Finally, preliminary experiments *in vitro* and *in vivo* suggested that the phenotypic differences observed in the OP9_F sample correlate with an improved hematopoietic potential of the generated progenitors. Indeed, CD34+ cells isolated from either OP9 (OP9_CTRL) or OP9 expressing human Flt3L (OP9_F) co-cultures had the unique ability to give rise to human CD45+ hematopoietic cells *in vivo* in MS5_FS12 stromal niches (Figure 4-5). Likewise, only the cells generated in OP9_F were capable of differentiate into CD14- CD1c+ cells when cultured *in vitro* on MS5_FST12 (Figure

4-4). Of note, in this *in vitro* experiment MS5_FST12 were used, even though they were later identified as sub-optimal for DC generation. However, the observed results were consistent with the differentiation potential described for this line in cord blood-derived HSPCs experiments (Figure 3-2). Nevertheless, further characterisation is needed to clearly establish the identity of these CD73- CD43+ CD41+ CD90+ cells as well as their differentiation potential.

3.1 Characterisation of *in vitro* generated CD73- CD43+ CD41+ CD90+ hematopoietic progenitors

The main effect observed in the iPSC-derived hematopoietic progenitors generated in OP9_F stromal cells is the differentiation of a unique subset of cells characterised by the expression of the hematopoietic markers CD43, CD41 and CD90 within the CD73- non-endothelial progenitors population. As previously mentioned, this phenotype is consistent with multipotent progenitors detected both in human cord blood samples as well as in iPSC-derived "HSC-like" cells, displaying a superior hematopoietic potential[623,671,672]. However, the absence of reliable extracellular markers to discriminate between primitive and definitive hematopoietic progenitors and the lack of a broad consensus on the phenotype of PSC-derived definitive cells represent a potential limitation of such approach. Therefore, a more extensive characterisation of the gene expression profile of these cells may allow a better understating of their identity and potential. In this regard, gene expression analysis by qPCR was performed in bulk populations of cells differentiated in engineered OP9 stromal cell (Figure 4-1 and Figure S4-1). This preliminary analysis highlighted that cells generated in OP9_F expressed significantly higher levels of SOX17, a transcription factor involved in the specification of hemogenic endothelium and proposed mark the appearance of definitive progenitors in PSC-derived hematopoietic differentiation[569,594]. Moreover, other

hematopoietic-related transcription factors, such as PU1, MYB and RUNX1, appeared to be modulated in these experiments, even though the observed differences were not significant (Figure 4-1). However, their up-regulation might be underestimated due to the massive dilution of this rare subset among other iPSC-derived hematopoietic and non-hematopoietic cells.

For this reason, future experiments will aim at assessing the expression of specific genes marking the onset of definitive hematopoiesis[597,623,645,648] by qPCR at single-cell level. The gene expression analysis of index sorted single cells will allow to correlate the gene signature characterising definitive hematopoietic cells with the phenotype of *in vitro* generated progenitors, enabling a more accurate assessment of their differentiation potential. Indeed, the most reliable confirmation of HSC emergence is the ability to give rise to all hematopoietic lineages including Type2 B cells, the only cell type exclusively originating from HSCs. This can be tested both *in vivo*, by injecting iPSC-derived progenitors in sub-lethally irradiated NSG mice and assessing their repopulation capability, and *in vitro*, by using stromal cell lines supporting hematopoietic differentiation (OP9 or OP9-DLL1, e.g).

Finally, an additional validation of the definitive origin of CD43+ CD41+ CD90+ cells can be achieved in genetically modified human iPSC, by CRISPR/Cas9-mediated abrogation of key transcription factors required for definitive hematopoietic cells development, such as MYB and RUNX1. It is anticipated that the lack of MYB or RUNX1 will impaired the generation of definitive progenitors in iPSCs co-cultures with OP9 and OP9_F, and the disappearance of the CD43+ CD41+ CD90+ cells would therefore confirmed their definitive nature.

3.2 Human Flt3L and SCF modulate iPSC-derived vascular endothelium specification

The expression of CD73 in iPSC-derived CD34+ cells clearly separates the endothelial-committed progenitors from cells displaying hematopoietic potential, including the hemogenic endothelium. Furthermore, the expression of CD184 (CXCR4) can be use to distinguish from arterial (CD184+) and venous (CD184-) vascular endothelium within the CD73+ endothelial cells[539].

Therefore, the effect of human cytokines expressed by engineered OP9 stromal cells on the specification of the iPSC-derived vascular endothelium was assessed *in vitro*. Flow cytometry analysis demonstrated that the presence of both human Flt3L and SCF affects the specification of CD73+ endothelial progenitors by promoting the differentiation of CD73+ CD184+ arterial vascular endothelium, which correlates with the reduction of the CD73+ CD184- venous counterpart (Figure 4-3b-c). Gene expression analysis of CXCR4 further supports this observation (Figure 4-3d).

In this regards, a further confirmation of the arterial and venous nature of CD73+ CD184+ and CD73+ CD184- cells can be achieved by assessing the expression of specific genes associated to arterial (EFNB2 and DLL4) and venous (NR2F2) vasculature by qPCR. Moreover, *in vivo* potential of these cells can be tested by subcutaneous injection of either CD73+ CD184- or CD73+ CD184+ cells in Matrigel plugs in NSG mice. The presence of large EPHB4- arterial vasculature or small EPHB4+ venous endothelium can be detected by immunostaining of histological sections of the explanted Matrigel grafts.

3.3 How does Flt3L affect hemato-endothelial development in human iPSC cultures?

A dual effect of human Flt3L in the development of both hematopoietic and endothelial cells *in vi*tro from iPSC was unravelled in this chapter (Figure 4-6). Indeed, the use of

OP9 stromal cells expressing Flt3L promoted the differentiation of CD43+ CD41+ CD90+ hematopoietic cells as well as the arterial specification in vascular endothelial cells. Therefore, the next question that needs to be answered is what are the mechanisms underlying Flt3L effect on hemato-endothelial development. One of the most interesting candidates that could participate in this process is the Notch signalling pathway. In fact, it has been recently demonstrated that Notch signalling is essential for endothelial-to-hematopoietic transition in human PSC-derived hematopoietic progenitors development[539]. On the other hand, the Notch pathway has been implicated in the regulation of vasculogenesis in different model organisms, and more importantly it has been shown to promote arterial specification of the vascular endothelium[539,673]. Hence, based on these observations, it is possible to hypothesise that Flt3L may promote indirectly the activation of Notch signalling pathway, which would explain the improved hematopoietic differentiation as well as the arterial vasculature induction. In this regard, a simple experiment to verify this hypothesis will consist in the use of Notch specific inhibitors, such as GSI (gamma-secretase inhibitors), in iPSC co-cultures with OP9 expressing human Flt3L (OP9_F). The abrogation of Flt3L-mediated modulation of both endothelial and hematopoietic development would confirm the involvement of Notch signalling in this process.

On the other hand, a second relevant aspect that requires clarification is the identification of the receptor mediating the Flt3L-depended effect observed. Indeed, preliminary experiments failed to detect both intra- and extra-cellular expression of Flt3 in iPSC-derived CD34+ cells (Figure S4-3a-b), and this was further confirmed by Flt3 expression analysis by qPCR (Figure S4-3c). However, Flt3 and Flt3L expression has been reported in the early phases of embryonic development in the IAHCs and surrounding endothelium in human AGM[466]. Therefore, even though these preliminary

observations will require further confirmation, alternative scenarios can be proposed. In this regard, the involvement of the VEGF signalling pathway may represent an interesting option. Indeed, VEGF has been identified as an essential regulator of Notch-dependent arterial specification in mice[674], and VEGFR2 (KDR) binding triggers the expression of both Notch and its ligand DLL4[575,675,676]. Moreover, VEGFR2 (KDR) is highly expressed in the early mesoderm as well as in hemogenic and non-hemogenic endothelial cells in human iPSC differentiation *in vitro*. Therefore, a first hypothesis may be based on the presence of Flt3L-responsive cells capable of secreting human VEGF, which signals through VEGFR2 receptor inducing Notch activation. A simple evaluation of the presence of human VEGF in the supernatant of iPSC co-cultures with OP9_F by ELISA may provide a first answer to this question.

Alternatively, a second explanation might rely on the cross-reactivity of Flt3L with other members of the receptor tyrosine kinases (RTK) family. In this regard, VEGFR2 (KDR) represents an interesting potential candidate.

The generation of human iPSC lines knockout for Flt3 may provide a very useful genetic model to assess the involvement of this receptor in the modulation of both hematopoietic and endothelial differentiation from iPSC *in vitro*, and the same approach can be transfer to other member of the RTK family.

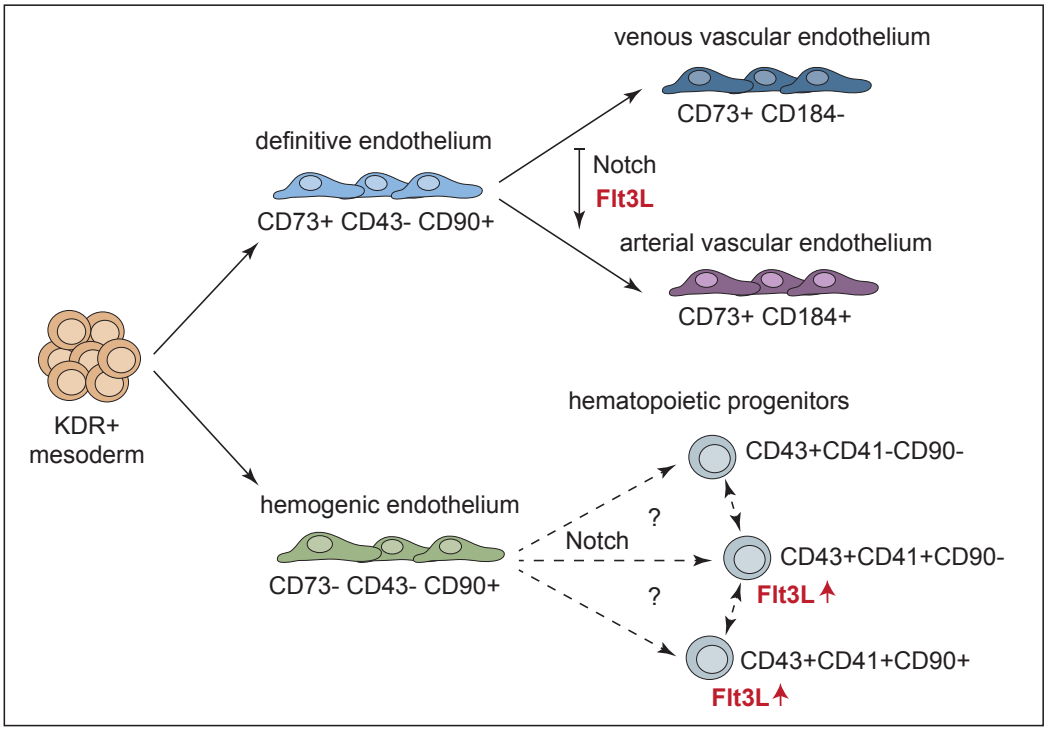


Figure 4-6. Effect of human Flt3L on hematopoietic and endothelial differentiation.

4 Supplementary figures

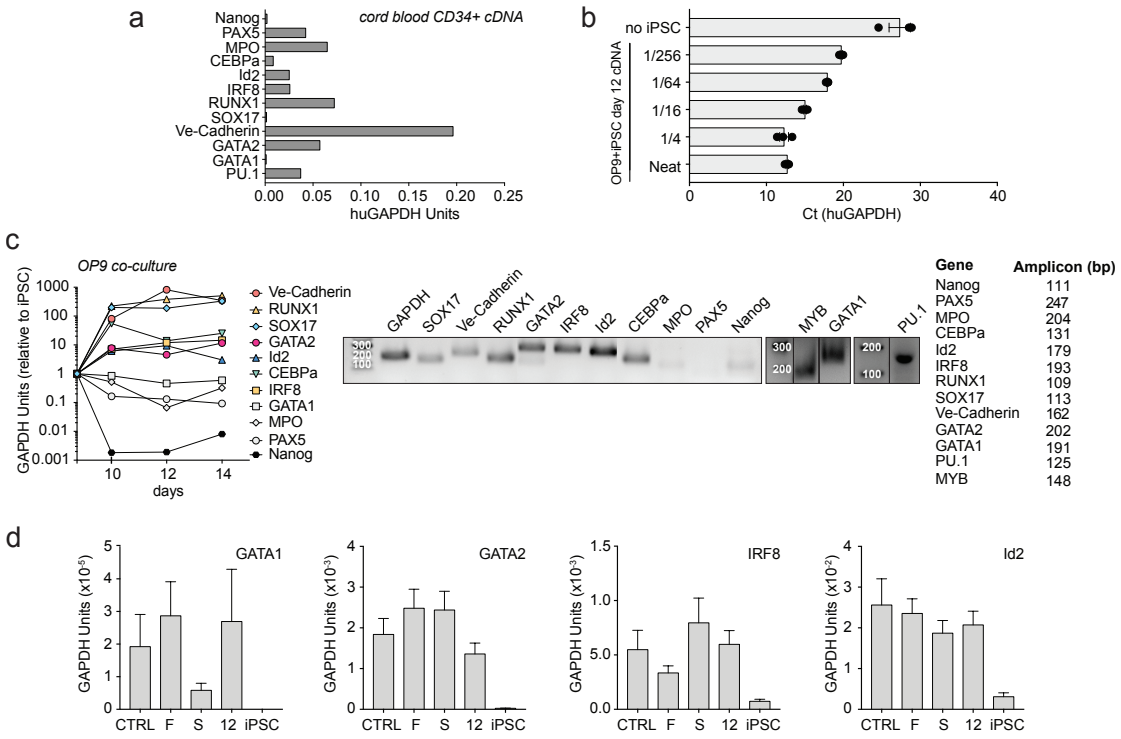


Figure S4-1. OP9 stromal cells expressing human Flt3L induce SOX17 expression in cells differentiated from human iPSC. (a) Positive control of qPCR primers using mRNA extracted from cord blood-derived CD34+ progenitors. **(b)** Detection of human GAPDH expression from serial dilution of mRNA extracted from bulk population of cells from day 12 co-cultures of human iPSC with stromal OP9 cells (n=3 technical replicates). **(c)** Expression profile of hematopoietic and endothelial genes in cells obtained from OP9 co-cultures as compared to undifferentiated iPSCs (left). Assessment of qPCR primers specificity by gel electrophoresis (right). **(d)** Gene expression analysis by qPCR of cDNA samples obtained by reverse transcription of mRNA extracted from bulk cell populations resulting from 12 days of iPSC co-culture with engineered OP9 stromal cells (n=7 technical replicates from 6 independent experiments using two iKCL4 and iKCL11 iPSC lines).

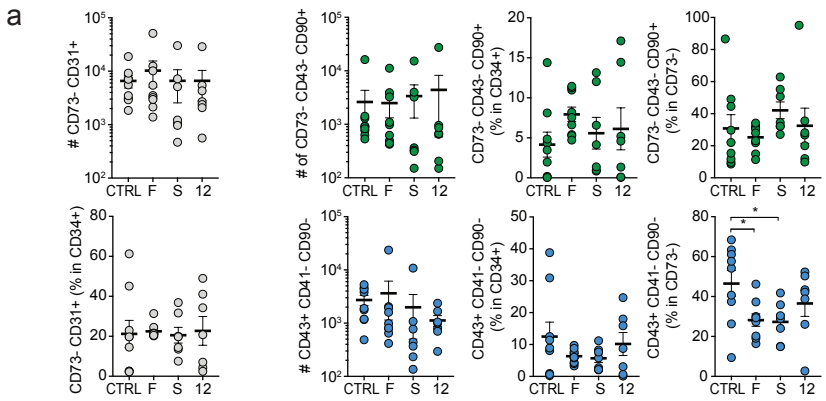


Figure S4-2. OP9 stromal cells expressing human Flt3L exclusively induce the differentiation of CD43+ CD41+ CD90+ hematopoietic cells from human iPSCs. (a) Quantification of additional subpopulations from figure 2d, expressed as absolute number of cells, frequency of CD34+ cells and frequency of CD73- CD31+ cells (n=7-9 technical replicates from 4 independent experiments using two iPSC cell lines. Mean ± SEM; * $p<0.05$, one-way ANOVA test).

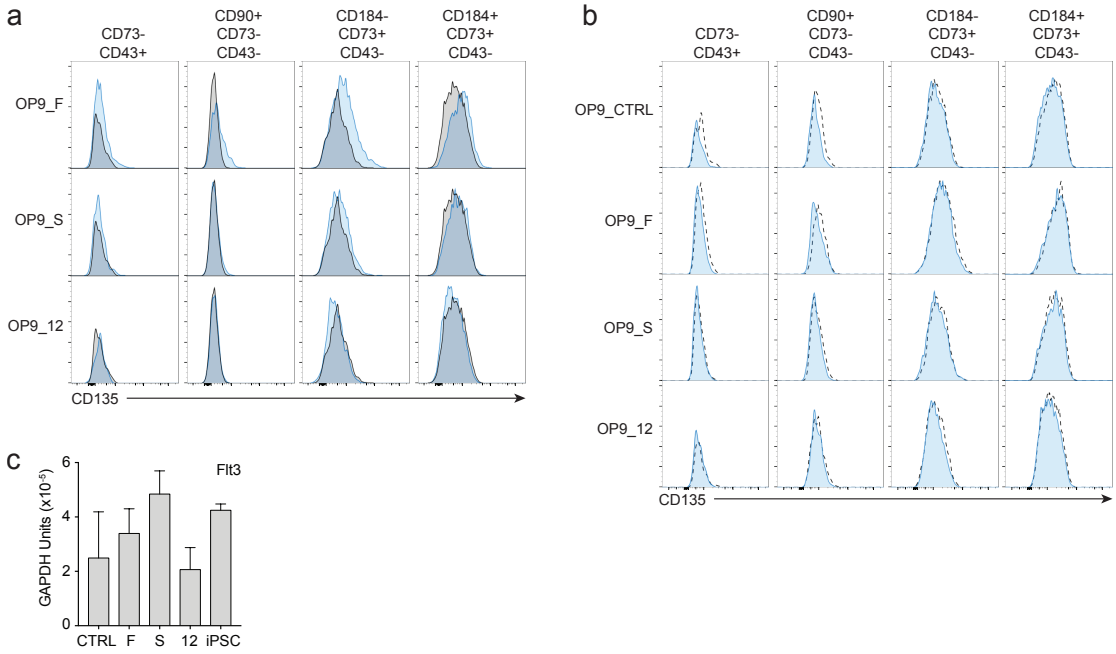


Figure S4-3. Flt3 expression in iPSC-derived CD34+ cells. Analysis of Flt3 (CD135) expression in iPSC-derived hematopoietic and endothelial cells by extracellular (a) and intracellular **(b)** flow cytometry (n=1). **(a)** Overlay of Flt3 expression in subsets generated from OP9_F, OP9_S and OP9_12 co-cultures (blue) as compared to OP9_CTRL (grey). **(b)** Intracellular expression of human Flt3 in subsets generated from OP9_CTRL, OP9_F, OP9_S and OP9_12 (blue) as compare to isotype control (dotted line). **(c)** Gene expression of Flt3 in cDNA samples obtained by reverse transcription of mRNA extracted from bulk cell populations resulting from 12 days of iPSC co-culture with engineered OP9 stromal cells (n=7 technical replicates from 6 independent experiments using two iKCL4 and iKCL11 iPSC lines)

Chapter 5 Genome-editing of human iPSCs

1 Introduction and objectives

The establishment of a reliable protocol supporting the differentiation of human dendritic cells from iPSCs would represent a major improvement in the understanding of human DCs biology. Indeed, iPSCs can be genetically manipulated by genome-editing techniques, such as CRISPR/Cas9, enabling the study of human DCs in a genetically tractable environment.

The understating of the transcriptional network driving DCs development is mainly based on observations from genetically modified mouse models[93,219,220], and subset-specific transcription factors driving DCs differentiation have been identified in the mouse. However, the current understanding of the transcriptional regulation of human DC is based on observations from patients harbouring genetic mutations in key transcription factors involved in the development of the mononuclear phagocyte system[71,236]. Therefore, testing the potential of genetically modified iPSCs to differentiate into human DC subsets would represent an invaluable approach to further identify essential factors involved in DCs development. Furthermore, the same strategy may be used to modulate dendritic cells immunogenicity as well as tolerance, by targeting specific pathways involved in either process. For instance, an extremely interesting source of potential targets is represented by the variety of factors known to modulate DCs immunogenicity as well as their ability to efficiently induce a tumor-specific cytotoxic T cell response[309]. If successful, this approach may not only shed new light on the mechanisms involved in the immune response against malignancies, but it could also represent a novel approach for the therapeutic vaccination against cancer.

1.1 Objectives

The aim of this chapter is the generation and validation of human iPSC lines knockout for known and unknown transcription factors involved in dendritic cells development as well as immune regulatory checkpoints using CRISPR/Cas9 system.

Therefore, the objectives of this section were:

- to modulate iPSC-derived DCs immunogenicity by targeting PD-L1 at the iPSC level;
- to generate clonal populations of human iPSC knockout for known and unknown transcription factors regulating human DC development including ID2, E2-2, IRF8, IRF4 and GATA2;

2 Results

2.1 CRISPR/Cas9-mediated PD-L1 knockout in human iPSC

In order to generate an iPSC line knockout for human PD-L1, the efficiency of different version of CRISPR/Cas9 vectors was first tested in the human melanoma cell line SK-MEL-28. SK-MEL-28 cells were chosen for their ability to express human PD-L1 in response to IFN-γ exposure, allowing the quantification of the impaired protein expression by flow cytometry. These preliminary experiments aimed at identify the more efficient CRISPR/Cas9 vector as well as validate the single-guide RNA designed to target human PD-L1.

Single guide RNA (sgRNA) targeting exon 1 of the genomic sequence of human PD-L1 was identified using the CRISPR design tool from the Zhang Lab (http://crispr.mit.edu). Complementary oligos carrying the 20nt sequence specific for human PD-L1 were annealed and cloned into three different versions of the lentiviral-based CRISPR/Cas9 vector: LentiCRISPR v1, LentiCRISPR v2 and the two-vector system LentiGuide-

Puro/LentiCas9-Blast (Figure 5-1a and Figure S5-1a). Lentiviral particles were generated and used to transduce the human melanoma SK-MEL-28 cell line. Seven days post-transduction the expression of PD-L1 in response to IFN-γ stimulation was measured by flow cytometry, highlighting an initial down-regulation of the protein in the cells co-transduced with LentiGuide-Puro (PDL1) and LentiCas9-Blast vectors (Figure 5-1b top and Figure S5-1b top). Transduced cells were then incubated in selection medium containing either Puromycin (LentiCRISPR v1 and LentiCRISPR v2) or a combination of Puromycin and Blasticidin (LentiGuide-Puro/LentiCas9-Blast) for seven days and the expression of PD-L1 was assessed by flow cytometry 16 days post-transduction. After selection, PD-L1 expression was abrogated in more than 60% of the cells transduced with LentiCRISPR v2 and LentiGuide-Puro/LentiCas9-Blast vectors. Conversely, only 7% of the cells targeted with LentiCRISPR v1 lack PD-L1 expression, which was completely unaffected in control cells transduced with the vector not expressing the 20nt sgRNA (Figure 5-1b bottom and Figure S5-1b bottom). Moreover, genomic DNA extracted from bulk population of cells targeted with the different vectors was analysed by Sanger sequencing in order to detect the presence of insertions or deletions (INDELS) in the targeted genomic region. The analysis of sequencing results showed that while 100% of the analysed samples (6/6) for LentiCRISPR v2 and LentiGuide-Puro/LentiCas9 targeted cells presented a 120bp and 167bp deletion, respectively, INDELS were detected in only 66% (4/6) of the samples targeted with LentiCRISPR v1 (Figure 5-1c and Figure S5-1c). Based on these results, the two-vector system LentiGuide-Puro/LentiCas9-Blast was selected for human iPSC targeting, and a line constitutively expressing Cas9 was generated and validated by Western Blot (Figure 5-1d). Cas9-expressing iPSC were then transduced with LentiGuide-Puro

(PDL1) vector and single-cell clones of Puromycin-resistant cells were isolated (Figure 5-1e).

Hence, an iPSC line constitutively expressing Cas9 was generated (iPSC-Cas9) and single-cell clones of iPSC targeted by a PD-L1-specific sgRNA were isolated. Future work will aim at validate the successful targeting in monoclonal populations by assessing the presence of genomic mutations by Sanger sequencing.

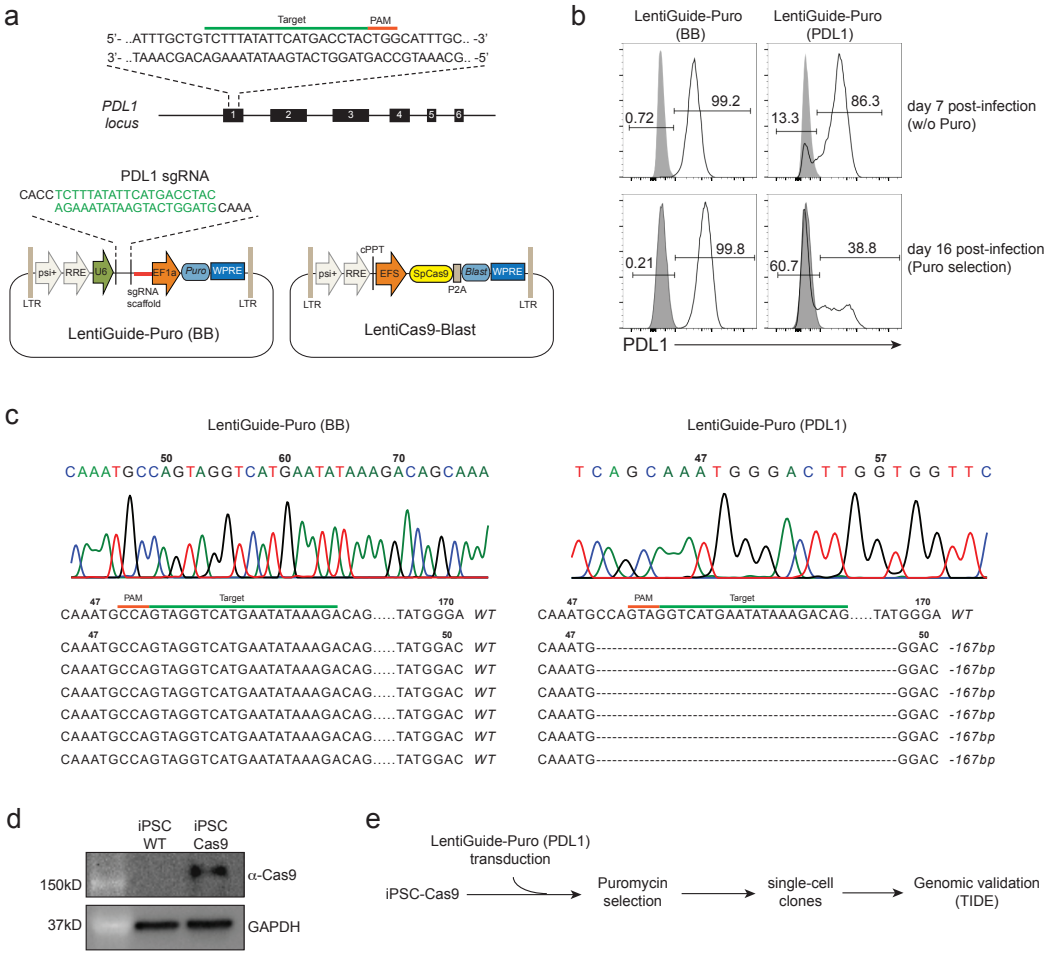
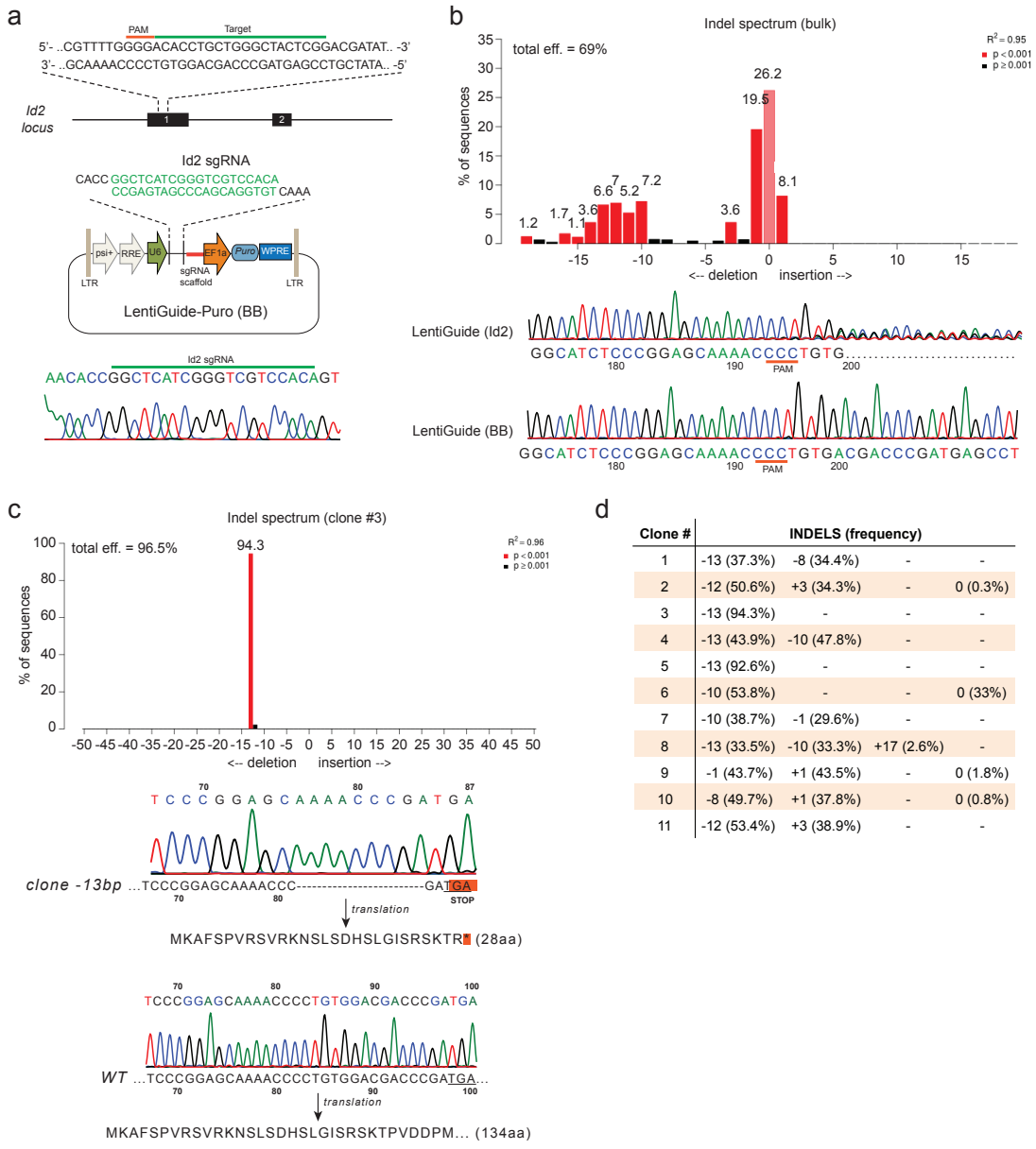


Figure 5-1. CRISPR/Cas9-mediated PD-L1 knockout in human iPSC. (a) Design of single guide RNA (sgRNA) targeting exon 1 of human PD-L1 gene and cloning into LentiGuide-Puro vector. **(b)** Evaluation of PD-L1 expression by flow cytometry in human melanoma cells (SK-MEL) transduced with LentiCas9-Blast vector along with LentiGuide-Puro in the presence (right) or absence (left) of the sgRNA targeting PD-L1 (right). Protein expression was evaluated before (top) and after (bottom) 7 days of puromycin selection. **(c)** Validation of PD-L1 targeting at genomic level in SK-MEL cells transduced with LentiGuide-Puro (PDL1) and the LentuGuide-Puro (BB) control. The presence of INDELS was assessed by Sanger sequencing. **(d)** Validation of an iPSC line constitutively expressing Cas9 by Western blot. **(e)** Experimental strategy to generate monoclonal populations of PD-L1 knockout iPSCs.

2.2 Generation of human iPSC lines knockout for transcription factors potentially involved in human DCs development

The abrogation of transcription factors potentially involved in human DC development can be achieved in iPSC by genomic editing using a CRISPR/Cas9-based approach. Therefore, based on the knowledge acquired from the mouse model as well as from human patients affected by DC deficiency candidate genes were identified, including the transcription factors IRF8, IRF4, GATA2, Id2 and E2-2. Single-guides RNA were designed for each factor using the CRISPR design tool from the Zhang Lab (http://crispr.mit.edu) and subsequently cloned into the LentiGuide-Puro vector as reported in Figure 5-2a, Figure 5-3a and Figure S5-2a. Two of these vectors, namely LentiGuide (IRF8) and LentiGuide (Id2), were used to generate lentiviral particles and transduced the previously established iPSC-Cas9 line (Figure 5-1d). After 7 days of Puromycin selection, the presence of INDELs was assessed using the online "tracking of indels by decomposition" (TIDE) tool form the van Steensel Lab [669] in bulk populations of both targeted lines. Decomposition analysis of the Sanger sequencing data demonstrated the presence of numerous mutations for both Id2 and IRF8-targeted cells, with a total efficiency of targeting estimated in 69% and 77.2% of the cells, respectively (Figure 5-2b and Figure 5-3b). The presence of inconsistent sequencing chromatograms in proximity of the expected double-strand break in the targeted samples as compare to the non-targeting controls LentiGuide (BB), further confirmed the efficient targeting of the expected genomic locus (Figure 5-2b and Figure 5-3b). Single-cell clones were subsequently isolated for each line, resulting in the establishment of 11 and 9 monoclonal populations of cells for Id2 and IRF8-targeted iPSC, respectively (Figure 5-2d and Figure 5-3d). Finally, TIDE analysis of Sanger sequencing data was performed in all the isolated clones for each cell line. Clone #3 of Id2-targeted iPSC was selected based on the prediction of a single -13bp deletion,

suggesting the presence of a homozygous mutation in the Id2 genomic locus (Figure 5-2c). *In silico* translation of the mutated genomic sequence allowed to identify the presence of an early stop codon resulting in a 28 amino acids (aa) truncated protein (Figure 5-2c).



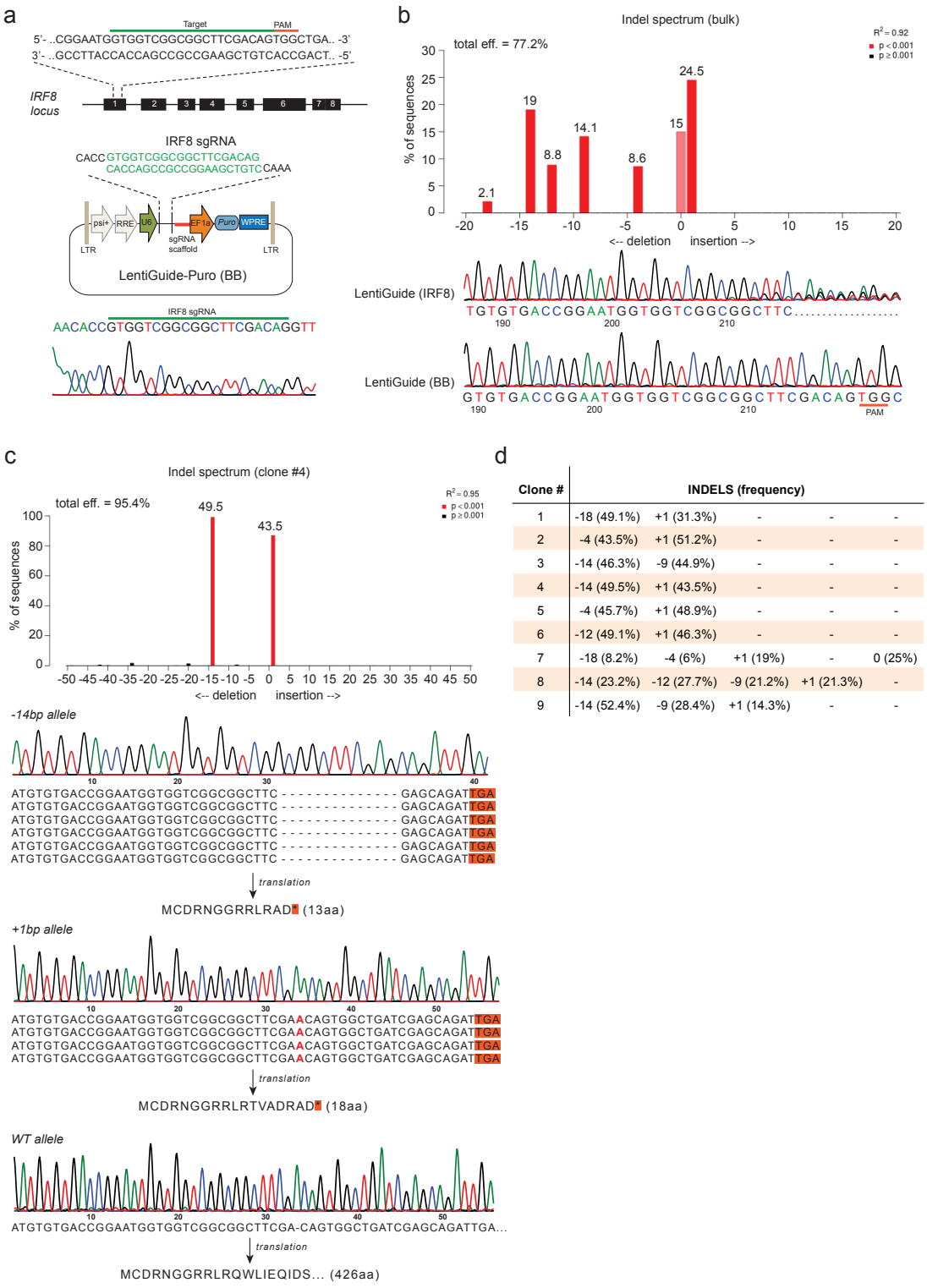
Clone #	INDELS (frequency)			
1	-13 (37.3%)	-8 (34.4%)	-	-
2	-12 (50.6%)	+3 (34.3%)	-	0 (0.3%)
3	-13 (94.3%)	-	-	-
4	-13 (43.9%)	-10 (47.8%)	-	-
5	-13 (92.6%)	-	-	-
6	-10 (53.8%)	-	-	0 (33%)
7	-10 (38.7%)	-1 (29.6%)	-	-
8	-13 (33.5%)	-10 (33.3%)	+17 (2.6%)	-
9	-1 (43.7%)	+1 (43.5%)	-	0 (1.8%)
10	-8 (49.7%)	+1 (37.8%)	-	0 (0.8%)
11	-12 (53.4%)	+3 (38.9%)	-	-

Figure 5-2. Generation of human Id2 knockout iPSC lines by CRISPR/Cas9 targeting. (a) Design of single guide RNA (sgRNA) targeting exon 1 of human Id2 gene and validation of the cloning into LentiGuide-Puro vector by Sanger sequencing. **(b)** Evaluation of the presence of INDELS in bulk population of Cas9-expressing iPSC transduced with the LentiGuide-Puro (Id2) vector after 7 days of puromycin selection. Sequencing data of the genomic region flanking the targeted site were analysed using TIDE-calculator [669]. **(c)** Evaluation of INDELS by TIDE-calculator analysis of Sanger sequencing results in a monoclonal population (clone #3) of iPSC targeted with LentiGuide-Puro (Id2) and *in silico* translation of the mutated genomic sequence predicting the insertion of an early stop codon. **(d)** Summary

of single-cell clones of CRISPR/Cas9-targeted iPSC and corresponding genomic mutations identified by TIDE.

In order to characterise the genomic mutations predicted by TIDE analysis, a heterozygous mutated clone (IRF8 #3) expected to carry a -14bp deletion and a +1 insertion was further analysed by Sanger sequencing. To identify the specific location of the described mutations for each allele, the targeted locus was amplify by PCR and amplicons were cloned into a donor vector (pUC19). Chemically competent bacteria (*E.Coli* DH10) were then transformed with the pUC19 vectors carrying either one of the two genomic sequences corresponding to two mutated alleles. The analysis of 10 bacteria colonies enabled the precise identification of the two mutations characterising clone #3 of IRF8-targeted cells, and allowed the *in silico* prediction of early stop codon insertions resulting in a 13aa truncated protein for the -14bp allele and a 18aa protein for the +1 allele (Figure 5-3c).

In conclusion, using a lentiviral-based two-vector CRISPR/Cas9 system, a Cas9-expressing iPSC line was transduced with sgRNA targeting human Id2 and human IRF8. Monoclonal population of cells were isolated, and two cell lines knockout for Id2 and IRF8 were established and validated by genomic sequencing of the targeted locus.



Clone #	INDELS (frequency)				
1	-18 (49.1%)	+1 (31.3%)	-	-	-
2	-4 (43.5%)	+1 (51.2%)	-	-	-
3	-14 (46.3%)	-9 (44.9%)	-	-	-
4	-14 (49.5%)	+1 (43.5%)	-	-	-
5	-4 (45.7%)	+1 (48.9%)	-	-	-
6	-12 (49.1%)	+1 (46.3%)	-	-	-
7	-18 (8.2%)	-4 (6%)	+1 (19%)	-	0 (25%)
8	-14 (23.2%)	-12 (27.7%)	-9 (21.2%)	+1 (21.3%)	-
9	-14 (52.4%)	-9 (28.4%)	+1 (14.3%)	-	-

Figure 5-3. Generation of human IRF8 knockout iPSC lines by CRISPR/Cas9 targeting. (a) Design of single guide RNA (sgRNA) targeting exon 1 of human IRF8 gene and validation of the cloning into LentiGuide-Puro vector by Sanger sequencing. **(b)** Identification of INDELS in bulk population of iPSC-Cas9 cells transduced with LentiGuide-Puro (IRF8) after 7 days of puromycin selection by TIDE analysis of Sanger sequencing data. **(c)** Evaluation of INDELS by TIDE-calculator analysis of sequencing results in a monoclonal population (clone #4) of iPSC targeted with LentiGuide-Puro (IRF8) and *in silico* translation predicting the consequences of the identified mutation at the protein level. **(d)** Summary of

single-cell clones of CRISPR/Cas9-targeted iPSC and corresponding genomic mutations identified by TIDE.

3 Supplementary figures

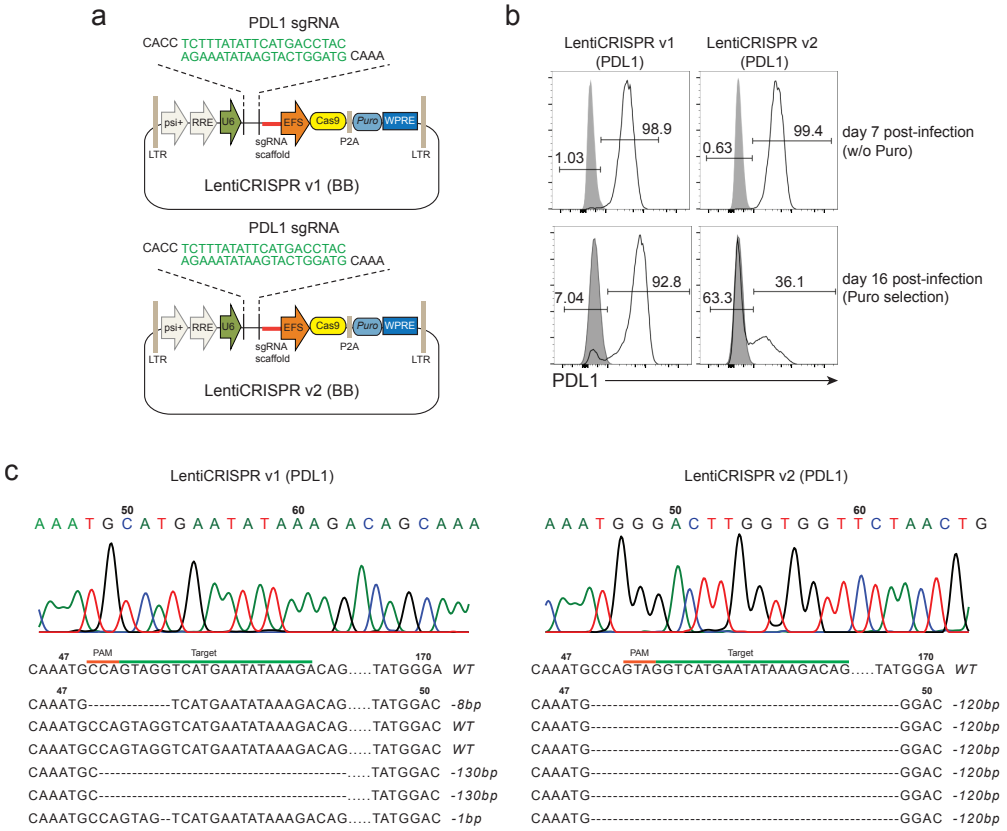


Figure S5-1. CRISPR/Cas9-mediated PD-L1 knockout in human iPSC. (a) Strategy to clone human PD-L1 sgRNA into LentiCRISPR v1 and LentiCRISPR v2 lentiviral vectors. **(b)** Evaluation of PD-L1 expression by flow cytometry in human melanoma cells (SK-MEL) transduced with LentiCRISPR v1 (PDL1) (left) and LentiCRISPR v2 (PDL1) (right) vectors, before (top) and after (bottom) 7 days of puromycin selection. **(c)** Validation of PD-L1 targeting at genomic level by Sanger sequencing, in SK-MEL cells transduced with LentiCRISPR v1 (PDL1) and LentiCRISPR v2 (PDL1) after puromycin selection.

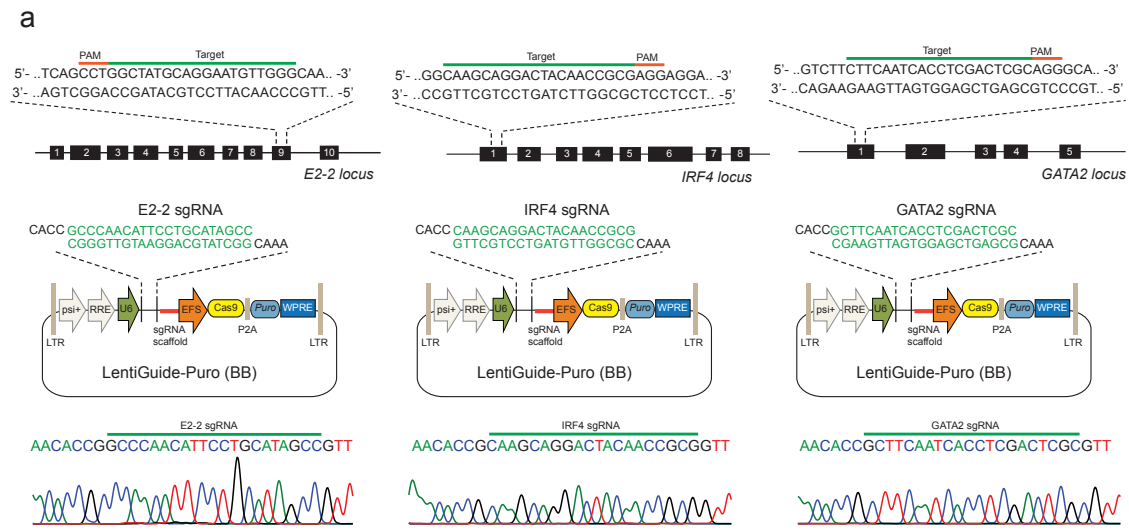


Figure S5-2. Generation of LentiGuide-Puro vectors targeting human E2-2, IRF4 and GATA2 genes. (a) Design of sgRNA specific for human E2-2, IRF4 and GATA2 and validation of the successful cloning into LentiGuide-Puro vector for CRISPR/Cas9 targeting in iPSC.

Chapter 6 Overall discussion and conclusions

The main goal of this project was to recapitulate the development of human dendritic cells *in vitro* from induced-pluripotent stem cell. To this end, the overall process was split in two independent steps: a first specification of definitive hematopoietic progenitors from iPSC and a second phase in which HSPCs were induced to differentiate into human DCs.

In order to define the best conditions supporting DCs differentiation, cord blood-derived hematopoietic progenitors were use in *in vitro* experiments.

Human dendritic cells arise from a common progenitor (CDP) residing in the hematopoietic niche in the bone marrow. In order to recapitulate the niche microenvironment *in vitro*, engineered mouse stromal cells (MS5) expressing the human growth factors Flt3L, SCF, CXCL12 and TPO were generated (Figure S3-1). Numerous combinations of factors were screened based on their ability to support DCs differentiation from cord blood-derived hematopoietic progenitors. As a result, MS5 expressing human Flt3L, SCF and CXC12 (MS5_FS12) were identified as the best condition to promote DCs development *in vitro* (Figure 3-2). Two main observations arose from the comparison of the different stromal lines generated: i) GM-CSF is not required for human DCs development; ii) TPO is a negative regulator of human DC1 and pDC differentiation. Moreover, MS5_FS12 were compared to the more commonly used protocol based on MS5 supplemented with recombinant Flt3L, SCF and GM-CSF (MS5+recFSG). As a result, MS5_FS12 yielded a significantly higher amount of cells for all the analysed subsets (DC1, DC2 and pDC). More interestingly, MS5_FS12 were also capable of maintain CD34+ progenitors to an undifferentiated state up to 15 days of culture (Figure 3-5).

More extensive analysis of the DC2 subset revealed that CD1c+ cells generated *in vitro* using MS5_FS12 are heterogeneous. Indeed, three subsets of CD1c+ cells could be identified based on the expression of CD14 and CD206: CD14- CD1c+ CD206- cells, CD14- CD1c+ CD206+ cells and CD14+ CD1c+ CD206+ cells. In order to understand the physiological relevance of these cells, further characterisation was performed. The phenotype of CD14- CD1c+ CD206- cells aligns with *bona fide* DC2 detected in human blood. Conversely, none of the CD206+ subsets were present in the blood of healthy volunteers (Figure 3-3). All CD1c+ subsets were activated by TLR4 stimulation (LPS) and preliminary data suggest that functional TLR8 was expressed only in CD14- CD1c+ CD206- and CD14- CD1c+ CD206+ cells (Figure 3-3). Moreover, CD14- CD1c+ CD206- cells were not differentiated nor expanded when only GM-CSF was provided *in vitro* and *in vivo*, highlighting their exclusive dependence on Flt3L. On the contrary, CD206+ cells were induced more efficiently by GM-CSF both *in vitro* and *in vivo* (Figure 3-4 and Figure S3-4). All together these observations suggest that i) CD14- CD1c+ CD206- cells closely align with circulating human DC2 subset; ii) CD14- CD1c+ CD206+ and CD14+ CD1c+ CD206+ cells do not exist *in vivo* in blood at steady state and may have a monocytic origin. However, further characterisation of these subsets is needed and the assessment of their presence in peripheral tissues at steady state or during inflammation *in vivo* represents a very important question that needs to be addressed in future experiments.

The ability of MS5_FS12 stromal cells to support human DCs differentiation *in vivo* in NSG mice was also tested. The injection of stromal cells along with human CD34+ progenitors in subcutaneous Matrigel plugs enabled the formation of three-dimensional "organoids" in which human DCs differentiate (Figure 3-6). The main advantage of this approach consists in the availability of terminally differentiated human DCs *in vivo*

within 12 days of differentiation and with no irradiation. The next goal is to provide evidences of the functionality of the *in vivo* generated subsets and to test their ability to interact with other human cell types (T cells, tumors, e.g.). Once established, this approach will enable the study of human DCs responses in *in vivo* models of diseases, for instance in the context of infection or cancer.

The successful differentiation of human DCs from iPSC requires the generation of definitive hematopoietic progenitors *in vitro*. Several publications reported the generation of human DC-like cells, expressing markers or fulfilling functions that are consistent with their DCs identity. However, very little is known about the hematopoietic programs involved in the differentiation of iPSC-derived DCs. In this manuscript, an *in vitro* culture system based on mouse stromal cells (OP9) expressing human hematopoietic growth factors (Flt3L, SCF, CXCL12, i.e.) was used to promote the generation of definitive progenitors capable of differentiating into human DCs. In this regard, the expression of human Flt3L in OP9 stromal cells (OP9_F) was the only condition tested that appeared to affect the development of iPSC-derived HSPC. Gene expression analysis showed a significant up-regulation of the transcription factor SOX17 in bulk population of cells differentiated in OP9_F (Figure 4-1). Moreover, the presence of Flt3L induced the differentiation of CD73- CD43+ CD41+ CD90+ cells, a phenotype consistent with multipotent progenitors displaying HSC-like potential (Figure 4-2)[623,671,672]. In addition, Flt3L expression promoted the differentiation of CD73+ CD184+ arterial vascular cells (Figure 4-3). Finally, iPSC-derived CD34+ cells from OP9_F co-cultures displayed an improved ability to give rise to human CD45+ cells *in vivo* in Matrigel plugs as well as to differentiate into CD14- CD1c+ cells in MS5_FST12 *in vitro* (Figure 4-4 and Figure 4-5). The improved hematopoietic

differentiation and the arterial vasculature induction are consistent with a potential activation of the Notch signalling pathway, a key regulator of definitive hematopoiesis. All together these observations may support the hypothesis that human Flt3L expression is capable of promoting the activation of the hematopoietic definitive program. Therefore, a more extensive characterisation of the gene expression profile and differentiation potential of cells generated in OP9_F is needed, to validate or refute this hypothesis.

In conclusion, two aspects characterising the differentiation of human DCs from iPSC have been investigated in this project. On one hand, a reliable system to support the differentiation of DC subsets from hematopoietic stem and progenitors cells has been established both *in vitro* and *in vivo*.

Moreover, a novel effect of human Flt3L in the early events of human embryonic hematopoiesis has been described. Preliminary evidences may suggest that Flt3L positively regulates the induction of a definitive hematopoietic program, even though further validation is required.

Finally, human iPSC lines knockout for DC-related transcription factors as well as the immune regulatory molecule PD-L1 have been generated using CRISPR/Cas9.

Future work will aim at merging together all the different aspects investigated in this project, in order to achieve the differentiation of human DCs from iPSC.

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